Faculty of Graduate Studies

Non target effect of pesticide on cultured rhizospheric bacterial communities of leguminous plants :*Vicia faba*

التأثير غير المباشر للمبيدات على المجتمعات البكتيرية المستنبتة حول المنطقة الجذرية للنباتات البقولية: الفول البلدي

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Non target effect of pesticide on cultured rhizospheric bacterial communities of leguminous plants: *Vicia faba*

By

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Declaration

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The work provided with this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: 8th June 2019

Dedication

This study has wholeheartedly dedicated to the Almighty God, thank you for the guidance, strength, power of the mind, protection, and skills and for giving me a healthy life.

To my beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continuously provide their moral, spiritual, emotional, and financial support, the individuals that have always pushed for success and great achievements, all credit of what I have achieved till this day goes to them.

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"Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time."

– Thomas A. Edison

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List of Abbreviations

- ACES: (*N*-[2-acetamido]-2-amino-ethanesulfonic acid),
- ANOVA: Analysis of Variance
- BCA: biological control agents
- BH: Bogiron high dose
- BL: Bogiron low dose
- C: Control
- CFU: Colony forming unit
- CH: Confidor high dose
- CL: Confidor low dose
- DH: Durspan high dose
- DL: Durspan low dose
- dRSM: Dilute rhizosphere medium (RSM)
- EC: Emulsifiable concentrate
- GC–MS: Gas Chromatograph(y) - Mass Spectrometry
- H_2S: Hydrogen sulfide
- IPM: Integrated pest management
- N_2: Nitrogen
- N_2 fixing bacteria: Nitrogen fixing bacteria
- PGPM: plant growth promoting microorganisms
- PGPR: Plant growth-promoting rhizobacteria
- PSB: Phosphate solubilizing bacteria
- RIM: Rhizosphere isolation medium
- SC: Suspension concentrate

1. Abstract:

Pesticides are amongst the most widespread pollutants; they have a diverse effect on the organisms in the environment such as plants, humans, animals, and microorganisms. Even though the use of pesticides increases overall yield, their misuse can lead to their accumulation in the environment which could affect non-target organisms such as rhizospheric bacteria. The present study aims at evaluating the non-target effects of selected pesticide son growth parameters, and on the cultured rhizospheric bacterial community of *Vicia faba* plants, together with assessing the effect of pesticides on cultured N_2 fixing bacteria.

Soil was treated with the common 3 pesticides (Confidor (Imidacloprid), Bogiron (Difenoconazole) and, Durspan (Chlorpyrifos)) used by Palestinian farmers to assess the changes in the microbial community of the rhizosphere at two time points (45 and 75days post-treatment) using two dosages (low dose and high dose). The residual concentrations of pesticides in soil were measured by GC–MS and the technique of enumeration of specific groups of rhizospheric microbes was employed by using two different media (RIM, dRSM); the presence of N_2 fixing bacteria was carried out by inoculation on nitrogen free medium. Cultured bacteria on RIM and dRSM were isolated and identified according to colony morphology and biochemical testing.

Plant parameters were not affected significantly by Confidor and Bogiron compared with the control, whereas Durspan affected both plant roots, which appeared thicker than control roots, and plant leaves, which presented brown dots on the surface. Moreover, roots' length was significantly decreased (18.67 cm shorter, p= 0.031) after the application of Durspan after 75 days when compared to the control.

The three pesticides studied differed widely in their soil degradation behavior, where Bogiron showed a slower degradation than Confidor and Durspan.

The average colony forming unit (CFU) counting of cultured rhizospheric bacteria on both media was different between both time points of post pesticide treatments for all treatments, and there was significant difference for same treatments between both doses. All treatments showed a significant decrease in the CFU at the first time point (Confidor low dose (3.03E+07 less<0.001), Confidor high dose (1.44E+07 less, p=0.044), Bogiron low dose (2.45E+07 less, p<0.001), Bogiron high dose (3.25E+07 less, p=0.000), Durspan low dose (3.29E+07 less, p<0.001), Durspan high dose (2.85E+07 less, p<0.001)) when compared with the control. The decrease in the CFU at the first time point is no longer detected at the second time point for all treatments with no significant change compared to the control. The data showed that the application of low dose of Confidor (1.92E+08 more, p=0.006), and Bogiron (2.61E+08 more, p<0.001), at both time points increased the growth of cultured rhizospheric bacteria.

There was a significant reduction in N_2 fixing bacterial groups observed after all the pesticide treatments at the first time point (Confidor low dose (1.33E+07 less, p=0.051), Confidor high dose (2.00E+07 less, p=0.006), Bogiron low dose (1.81E+07 less, p=0.011), Bogiron high dose (1.79E+07 less, p=0.012), Durspan low dose (2.42E+07 less, p=0.002), Durspan high dose (2.24E+07 less, p=0.003)) compared with the control with maximum reduction associated with the application of Durspan. At the second time point there was no significant difference after application of both doses of Confidor and Bogiron compared to the control, but a significant reduction after application of both dosages of Durspan (3.82E+07 less, p=0.014).

Thirty-nine separate and different colonies were counted on both media (RIM, and dRSM) which were isolated and identified based on their morphological characteristics and biochemical tests.

Twelve isolates were confirmed to belong to *Pseudomonas sp*, four isolates were identified to belong to *Actinobacteria sp* according to their morphology, three isolates were confirmed to belong to *Bacillus sp* (two of them *Bacillus cereus* and one *Bacillus subtilis)*. Two isolates were identified as *Staphylococcus sp* according to their morphology and cell staining, the remaining 18 isolates still need to be identified. Most of the isolates are able to solubilize phosphate and fix nitrogen.

This study indicates that pesticides show non-target effects on active microbial populations that attend important ecosystem functions by enhancing or inhibiting their growth. Confidor and Bogiron enhance the growth of cultured rhizospheric bacteria with time, while Durspan seems to inhibit the growth of cultured rhizospheric bacteria with time. These results highlight the need to further study and understand of how the use of pesticides in agriculture can affect the environment, and the need to establish guidelines for their use. This study recommends avoiding the use of Durspan as insecticide on *Vicia faba*.

2. Introduction

Various studies have been done to evaluate the effect of pesticide on the diversity of soil microbes; these studies have been carried out to reproduce scenarios of massive pesticide pollution using excessive doses of pesticides, which are impractical and unlikely to occur on a regular basis (Gupta et al., 2013). With the increased use of pesticides in agriculture, it is very important to understand their impact on non-target soil microbial communities for the protection of soil health (Singh et al., 2015b). The degradation of pesticides is different according to the composition of the pesticide, and the residue of pesticide found in the soil can affect microbial communities (Gilani et al., 2010a).

A study done by Gilani, S., et al. on the degradation of the pesticide chlorpyrifos, followed the destiny of this pesticide six months and one-year after application and showed that no degradation occurred during the study period; 100% recovery of the active ingredient was observed after one year under lab conditions. This experiments highlighted that the growth of *Bacillus spp*. was sensitive to chlorpyrifos treatment i.e. the number of colonies recovered from soil after treatment decreased, whereas the number of colonies of *Klebsiella spp*. increased during the same treatment in the experiments (Gilani et al., 2010a). A study done by Fang, Yu et al. concluded that chlorpyrifos residues in soil had a temporary or short-term inhibitory effect on soil microbial functional diversity (Fang et al., 2009).

A study by Gupta et al. showed that pesticides have a non-target effects on active microbial populations which aid significant ecosystem functions (Gupta et al., 2013). Bacterial diversity seemed to decrease in soils treated with urea herbicides as shown by a study of El Fantroussi, S et al, in which treating plants with urea herbicides for a long period of time, resulted in marked

negative changes in both the metabolic and structure potential of soil microbial communities (El Fantroussi et al., 1999). Newman et al investigated shifts in the rhizosphere bacterial communities; in this case, the authors reported a decrease in abundance of *Acidobacteria* after application of glyphosate and speculated that this could result in a dramatic change of the rhizosphere nutritional status (Newman et al., 2016).

Ahemad and Khan carried out a study to measure the misuse of pesticide by assessing the impact of pesticide at different concentrations on pea plants (Ahemad and Khan, 2012). Their study was designed to evaluate the influence of pesticides: insecticides (imidacloprid), fungicides, and herbicides at the recommended and higher dose on plant growth stimulating behaviors of *Rhizobium spp*. that was isolated from pea-nodules. The maximum toxicity to plant growth promoting behaviors was shown at the higher dose rate (Ahemad and Khan, 2012). Another study by the same authors was planned to evaluate the consequence of certain pesticides (metribuzin, thiamethoxam, kitazin, glyphosate, metalaxyl, hexaconazole, and imidacloprid) at the same doses on plant growth promoting activities of *Pseudomonas aeruginosa* strain PS1 that was isolated from mustard rhizosphere. Plant growth promoting traits decreased linearly with increase of pesticide concentration (Ahemad and Khan, 2011). This study further underlines how a healthy rhizosphere is essential for good plant growth.

In an experiment to study non-target effects of difenoconazole (fungicide), and other pesticide on microbial parameters in a clay-loam soil carried out by Muñoz Leoz et al., pesticides wereapplied as commercial formulations to soil samples at different concentrations (5, 50, and 500 mg kg−1DW soil), then the microbial parameters were determined at days 7, 30, 60 and 90. At the lowest concentrations, none of the pesticides caused significant changes in soil microbial parameters. However, at 500 mg kg−1 DW soil, pesticide application decreased overall soil

microbial activity, negatively affecting the activity of soil enzymes. Also, at 500 mg kg−1 DW soil, difenoconazole caused a pesticide-induced stress on soil microbial communities, as reflected by the respiratory quotient. The study concluded that, although pesticide concentration had a somewhat inconsistent and erratic effect on soil microbial parameters, pesticide application at 500 mg kg−1 DW soil did have an impact on many of the microbial parameters considered (Muñoz-Leoz et al., 2013). Thomet al, examined the degradation of the fungicide difenoconazole in a silt loam soil under controlled conditions, and concluded that difenoconazole is metabolized by an acclimated part of the soil microflora. However, the degradation seems to be stimulated in the presence of suitable co-substrates (Thom et al., 1997).

The inhibitory effects of the pesticide Chlorpyrifos was studied by (Wang et al., 2010) through the measurement of metabolic parameters and the microbial urease enzyme; they concluded that when soil microorganisms were exposed to chlorpyrifos with different dose, microbial metabolic activities were suppressed at different rates, which illustrates that individual organisms reacted to stress from environment change by shifting resources from other biological activities (such as reproduction or growth) and toward survival. Another study by Sing et al.found that chlorpyrifos at the higher dosage inhibited *Pseudomonas spp*.(Singh et al., 2015a), while another study by Ahmed and Ahmad showed that chlorpyrifos caused significant reduction in number of soil bacteria(Ahmed and Ahmad, 2006) .

A study done by Walvekar et al. (Walvekar et al., 2017) shows that nitrogen fixers, which play a crucial role in making organic nitrogen available for plants, were the most deleteriously affected group upon pesticide application. The detrimental effect of pesticides on nitrogen fixation was confirmed by a study done by Sing et al. using cultivation-independent analysis (Singh et al., 2015a). This study concluded that the active bacterial community involved in nitrogen fixation

was disturbed toward the later stage of legume growth, as it recorded a reduced number of *nifH* transcripts with the application of chlorpyrifos at both doses. Another study done by Fox et al. shows that a one-time treatment with some natural and synthetic environmental chemicals is sufficient to significantly inhibit nodule formation and that nitrogenase activity was significantly reduced in all chemical treatment groups compared with the control(Fox et al., 2007). They suggested that this may be due to the fact that natural phytochemical and synthetic agrichemicals hinder the symbiotic signaling between host plants and neighboring bacterial community as shown in the rhizosphere of alfalfa plant.

It is therefore important to understand the effect that pesticides might have on the microbial communities associated with plants that are of important economic and nutritional value. In this study the effect of three pesticides on the rhizosphere of the broad bean *Vicia faba* is being evaluated. This study focuses on isolates from the rhizosphere and on the effect of pesticides on nitrogen fixing bacteria. The results indicate that pesticides show non-target effects on active microbial populations that attend important ecosystem functions in the rhyzosphere. Confidor and Bogiron enhance the growth of cultured rhizospheric bacteria with time, while Durspan seems to inhibit the growth of cultured rhizospheric bacteria with time.

3. Literature review

3.1 Legumes

Legumes are a known source of micronutrients and macronutrients. They have performed a significant function in the traditional diet of many regions around the world, and contain nutritional components that promote good health and can have therapeutic properties(Xu and Chang, 2007). Presence of legumes in the daily diet has many beneficial physiological effects in controlling and preventing various metabolic diseases such as diabetes mellitus, coronary heart disease, and colon cancer (Tharanathan and Mahadevamma, 2003)

Legumes are considered poor man's meat. They are generally good sources of slow release carbohydrates and are rich in proteins. Legumes are normally consumed after processing, which not only improves palatability of foods, but also increases the bioavailability of nutrients by inactivating trypsin, growth inhibitors, and hemagglutinins (Tharanathan and Mahadevamma, 2003). The legumes' by-products are valuable sources of functional compounds and might be an important source of dietary fiber. Dietary fiber, which is a heterogeneous mixture of several types of polysaccharides, is rich in legumes, especially in their husk fractions and contributes to beneficial therapeutic health effects (Tharanathan and Mahadevamma, 2003). Dietary fiber plays an important role in many physiological processes and in the prevention of diseases (Mateos-Aparicio et al., 2010). It is also reported that dietary fiber has potential for treatment of Parkinson disease, hypertension, renal failure, and liver cirrhosis (Randhir and Shetty, 2003, Rabey et al., 1992)

Legumes also have an important role in improving soil quality and possess all the characteristic properties of green manures such as: the ability to add a great amount of organic material and nutrients to soil, the ability to prevent leaching of nutrients and soil erosion, and the ability to

improve soil's capacity to hold water. The most important of these properties is their ability to influence the total nitrogen (N) economy of soil by repossessing atmospheric N through symbiotic N_2 fixation and through subsoil N retrieval (Sharma et al., 2005)

3.2 Fava bean (*Vicia faba)*

Fava bean is one of the oldest crops having long tradition of cultivation in the old world agriculture (Singh and Bhatt, 2012). Its most common use is as human food in developing countries (mainly Asia and Africa) (Duc, 1997). It is known by many names such as broad bean, horse bean, field bean, and Windsor bean (Singh and Bhatt, 2012, Duc, 1997). Globally, Fava bean (*Vicia faba*), is the third most important consumed feed grain legume (Singh and Bhatt, 2012, Singh et al., 2013). It is one of the crops with the most potential to serve humanity at global level; China is currently the world leading producer with 60% of total yield, followed by Northern Europe, the Mediterranean regions, Ethiopia, Central and East Asia, and Latin America (Singh et al., 2013). Fava bean is known to have been cultivated from the early Neolithic age. It can be said that it has been known from the beginning of agriculture (Cubero, 1974). The fava bean has Middle Eastern origins and centers of diversity in the Far East and Europe (Landry, 2014). It is as an agronomically viable alternative to cereal grains (Singh and Bhatt, 2012, Singh et al., 2013)

The cultivated forms of fava bean are grown in different agro climatic conditions depending upon the suitability of the strain to a particular area (Singh and Bhatt, 2012). It is an annual plant which requires cool conditions for best development. It is normally planted in the spring in northern latitudes, in the winter in warm-temperate and subtropical areas, but is also a popular crop at higher elevation (Singh and Bhatt, 2012, Duc, 1997, Singh et al., 2013). It can be grown in adverse soil conditions (soil pH), it is widely adapted to diverse soil types, and is more tolerant towards acidic

as well as saline alkaline soils than most legumes. Fava bean is susceptible to many pests and pathogens and to different abiotic stresses (Singh and Bhatt, 2012). The roots form endo-mycorrhizal associations. Stem growth is indeterminate, and results from the growth of two orthotics which alternatively develop a node, carrying a leaf up to the 5th to 10th node and thereafter carrying a raceme of 2 to 12 flowers axillary to the leaf. The flowers are 2-3 cm long at anthesis. They can be completely white, brown or violet. The pods in *minor* and *paucijuga* types are short and erected (3-4 ovules per pod) but in *major* types are long and hanging (8-12 ovules per pod). (Duc, 1997).

Fava bean plants establish symbiotic relationship with bacteria that are capable of fixing atmospheric nitrogen, which results in increased residual soil nitrogen for use by subsequent crops. It is one of the best annual crops which can be used as green manure having potential of fixing free nitrogen (100-350kg N /ha). (Singh and Bhatt, 2012, Landry, 2014). The benefits of fava beans to the soil include increased microbial diversity, soil sanitation, soil structure, and less water use (Landry, 2014).

3.3 Symbiotic relationship between leguminous plant and rhizosphere microorganisms

The majority of legume species can establish symbiotic associations with two important groups of rhizosphere microorganisms: bacteria of the genus *Rhizobium* and arbuscular mycorrhiza fungi of the order Glomales (Ahemad and Khan, 2013, Frühling et al., 1997). The term ‘‘rhizosphere’’ has been derived from the Greek word ‘rhiza’, meaning root, and ‘sphere’, meaning field of influence (Prashar et al., 2014). It was first defined by the German scientist Hiltner (1904) as ‘‘the zone of soil immediately adjacent to legume roots that supports high levels of bacterial activity”(Prashar

et al., 2014, Berg and Smalla, 2009, HILTNER, 1904). Over a period of time, the definition has been changed and redefined many times to encompass "the volume of soil influenced by the root and parts of root tissues as well as the soil surrounding the root in which physical, chemical and biological properties have been changed by root growth and activity" (Prashar et al., 2014, Sharma et al., 2005). This particular zone in agricultural soil is characterized by a distinct microbial community structure, which differs from bulk soil in its composition and activity. The rhizosphere has been regarded, therefore, as a hot spot for microbial colonization and activity. The microbes of the rhizosphere and their functions are highly influenced by the plant root, and in turn the bacterial and fungal members in the rhizosphere have a high impact on plant growth (Sharma et al., 2005). Several biotic and abiotic factors influence the structural and functional diversity of bacterial communities, for example: climate and season, grazers and animals, pesticide treatments, soil type and structure, and plant health and developmental stage (Berg and Smalla, 2009).Over time, the composition of bacterial communities changed with soil aging that developed over c. 77 000 years of intermittent Aeolian deposition, and the overall richness, diversity and evenness of the communities increased (Berg and Smalla, 2009).

The symbiotic relationship between leguminous plant and Rhizobia species inside the soil results into shared resources (Berg and Smalla, 2009, Yamal et al., 2016). The driving force for this process is the root exudates. The composition of root exudates varies from plant to plant and it selectively influences the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the area around the plant roots and by serving as selective growth substrates for soil microorganisms (Berg and Smalla, 2009, Yang and Crowley, 2000). Plants not only provide nutrients for microorganisms, but some plant species also contain unique antimicrobial metabolites in their exudates (Berg and Smalla, 2009). Because the root exudates consist of water,

ions, free oxygen, mucilage, enzymes, and a diverse array of carbon-containing primary and secondary metabolites, they are considered the key factor for the supplementation of specific microbial populations in the rhizosphere. From 10% up to 44% of the photosynthetically fixed carbon is discharged by the root (Berg and Smalla, 2009). Organic acids, sugars, amino acids, lipids, coumarins, flavonoids, proteins, enzymes, aliphatics, and aromatics are examples of the primary substances found at the soil–root interface (Berg and Smalla, 2009). The important role of the organic acids in providing substrates for microbial metabolism, and for serving as intermediates for biogeochemical reactions in soil, received significant attention. The amount and composition depend on the plant family or species (Berg and Smalla, 2009), and determine the structure and function of microbial communities in the rhizosphere (Sharma et al., 2005).

Microorganisms in turn influence the composition and quantity of various root exudate components through their effects on root cell leakage, cell metabolism, and plant nutrition. Based on differences in root exudation and rhizo-deposition in different root zones, rhizosphere microbial communities can vary in structure and species composition in different root locations or in relation to soil type, plant species, nutritional status, age, stress, disease, and other environmental factors(Yang and Crowley, 2000). The structure variation in microbial communities influences ecosystem processes (e.g., decomposition of organics, and nutrient recycling) and the interaction between plant and microbes (e.g., release of plant-growth promoting rhizo-bacteria, genetically engineered microorganisms or growth of pathogens); the understanding of how the community processes affect ecosystem processes has become a central interest in ecology(Berg and Smalla, 2009, Sharma et al., 2005, Antoun and Prévost, 2005).

The rhizosphere has been commonly subdivided into the following three zones:

1. Endorhizosphere: composed of the root tissue inclusive of the cortical layers and endodermis

2. Rhizoplane: the surface of the root where the microbes and soil particles adhere. It contains cortex, epidermis and a mucilaginous polysaccharide layer.

3. Ectorhizosphere: composed of the soil directly neighboring the root(Prashar et al., 2014).

3.4 Rhizosphere microorganisms

In the rhizosphere, the most abundant microorganisms are bacteria, and Rhizobacteria are the rhizosphere competent bacteria that heavily colonize around the plant roots. They can multiply and colonize in ecological niches on the roots at all stages of plant growth, even in the presence of a competing microflora (Prashar et al., 2014, Antoun and Prévost, 2005).

A variety of bacterial genera are found to cover up to 15% of the total root, including: *Pseudomonas, Bacillus, Arthrobacter, Rhizobia, Agrobacterium, Alcaligenes, Azotobacter, Mycobacterium, Flavobacter, Cellulomonas,* and *Micrococcus* (Prashar et al., 2014). There are microbial species that are naturally associated with only a few or a single plant species. For example, Rhizobia legumes: *Rhizobium leguminosarum* induces nodules in *Pisum vicea*, and *Lathyrus* plants. Another example is *Sinorhizobium meliloti* which effectively colonizes plant genera of *Medicago, Melilotus,* and *Trigonella*. Other microbial genera like *Pseudomonas* and the fungus *Trichoderma* are widespread, but it still probable to find new taxonomic groups in the rhizosphere. For example, non-thermophilic members that belong to the archaeal division *Crenarchaeota* (Berg and Smalla, 2009) are likely members of the rhizosphere microbial community. The bacterial strains that are predominant in the rhizosphere contain gram-negative, rod shaped, non-sporulating bacteria that belong to the group *Proteobacteria*, with *Pseudomonas* as the most common, and the gram positive *Actinobacteria* (Antoun and Prévost, 2005, Antoun et al., 1998). This may be because the effectiveness of the gram negative bacteria's consumption of

root exudates, combined with stimulation by rhizo-deposition, inhibits the growth of most gram positive bacteria (Steer and Harris, 2000).

Also the variety of aerobic bacteria is comparatively less because of the reduced oxygen levels in the rhizosphere due to root respiration. The reduction of oxygen doesn't affects the N_2 fixing bacteria because many nitrogen-fixing organisms fix nitrogen only under low oxygen atmospheric concentrations (Gallon, 1981). Those organisms that can fix nitrogen aerobically are usually badly affected by exposure to elevated concentrations of oxygen, because of its effect on nitrogenase activity and synthesis.(Gallon, 1981). The gram-positive, rods or cocci, and spore forming strains like *Bacillus* and *Clostridium* are relatively less numerous. Strains of *Bacillus* are considered the main inhabitants gram-positive bacteria of the rhizosphere (where up to 95 % of total gram-positive are soil bacilli followed by *Frankia* and *Arthrobacter* (Prashar et al., 2014, Antoun et al., 1998). The gram negative Rhizobia (including *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium*) are mostly observed as the partners for the microbial symbiosis with legumes and they are mostly regarded for their function in the formation of nitrogen-fixing nodules (Antoun and Prévost, 2005).

Beneficial rhizosphere organisms can be classified depending on their primary effects specifically on plants: (1) microorganisms with direct effects on plant growth promotion [plant growth promoting microorganisms (PGPM)] and (2) microorganisms with indirect effects as biological control agents (BCA) that indirectly contribute, together with plant products, to the control of plant pathogens (Whipps, 2004, Vassilev et al., 2006) .

PGPR rhizobacteria are free-living bacteria, some of them have the ability to attack the tissues of living plants and cause asymptomatic infections. These rhizobacteria are stated as endophytes, and in order to occupy the roots they must first be rhizosphere competent (Antoun and Prévost, 2005).

PGPR may induce plant growth by direct or indirect modes of action; direct mechanisms entail the production of phyto-hormones and stimulatory bacterial volatiles, liberation of phosphates and micronutrients from insoluble sources, and non-symbiontic nitrogen fixation, which improves the plant nutrient status, lowers ethylene levels in plants, and stimulate disease-resistance mechanisms (Antoun and Prévost, 2005, Antoun et al., 1998). Indirect mechanisms include the ability of PGPR to act like biocontrol agents reducing disease, to degrade xenobiotics in contaminated soils, or to stimulate other beneficial symbioses (Antoun and Prévost, 2005, Antoun et al., 1998). Further to their plant growth promoting effects, *Rhizobium spp*. have been increasingly associated with disease suppressive effects (Elbadry et al., 2006, Huang and Erickson, 2007). The disease suppression conferred by *Rhizobium spp*. has been linked to direct inhibition of pathogens development (through competition or antibiosis), as well as indirect inhibition through the stimulation of plant defense mechanisms (Avis et al., 2008). Additional example of biocontrol by *Rhizobium spp*. is their ability to produce iron-chelating siderophores that decrease or exclude accessible iron for other microorganisms in the same ecological niche and therefore generate antagonistic activity through competition (Dakora, 2003).

3.5 Nitrogen fixation symbiosis

The interaction between *Rhizobium*-legume leads to symbiotic nitrogen fixation which is carried out in a particular plant organ: the root nodule (Frühling et al., 1997). This symbiotic interaction is the best studied system of plant-microbe interactions (Vieweg et al., 2004, Perlick and Pühler, 1993). This symbiosis generates a bidirectional exchange of nutrients with inorganic nutrients moving to the plant, and carbon components flowing to the micro symbiont. The target tissue for the micro symbiont is the root cortex. Inside colonized root cells, the micro symbionts are

separated from the host cytoplasm by cell wall material and specific plant-derived membranes (Vieweg et al., 2004).

Nitrogen is considered an essential nutrient for the synthesis of a wide range of metabolites like proteins, chlorophyll, nucleic acids, and enzymes. It critically determines the health of all living organisms. Nitrogen is present in the atmosphere in a large amount (78%), and growing plants cannot consume it in gaseous form (N_2).

Nitrogen fixing microorganisms that exist in the root systems of leguminous plants have developed a strategy that converts the atmospheric N_2 into ammonia (biological N_2 fixation BNF) using the enzyme nitrogenase (Ahemad and Khan, 2013).

Inside the plant tissue, where nodule formation occurs, specific proteins, *nodulins*, are released during all stages of nodule formation (Perlick and Pühler, 1993, Yamal et al., 2016). The formation process of indeterminate nodules can be divided into the following developing stages: a) root hair curling: this is considered a pre-infection stage. The root ends curl and bacteria are still outside the plant cells. b) development of infection threads: the bacteria attack the root hairs with tubular infection threads created by cell wall materials and plant synthesized membranes. c) primordia induction and invasion: plant cells start dividing by mitosis and meristematic activity is stimulated independently in the root cortex that results in the formation of nodule primordia which leads to infection threads growth across these cells. d) invasion of cell cytoplasm: bacteria enveloped by the plant peri-bacteroid membrane, are released into the host cell cytoplasm. e) proliferation and differentiation: inside the plant cell the bacteria proliferate and differentiate into bacteroids capable of nitrogen fixation (Perlick and Pühler, 1993, Ahemad and Khan, 2013).

The exchange of N_2-fixing symbiosis happens in the rhizobia-infected cells of the nitrogen-fixing zone of the root nodule. (Vieweg, Frühling et al. 2004). Genes involved in the N_2-fixing symbiosis

have been subdivided into early and late *nodulin* genes, depending on the onset of their expression during nodule development (Vieweg et al., 2004). Early *nodulin* genes are expressed after mutual recognition between the plant and the rhizobia and before the start of nitrogen fixation. They are believed to play a role in the infection process and in nodule organogenesis (Frühling et al., 1997, Vieweg et al., 2004, Perlick and Pühler, 1993). The most prominent members of this group are certainly the*NOD2* proteins, which have been detected in all legumes analyzed so far. *NOD2* proteins are located in the nodule parenchyma and are thought to be involved in the formation or function of the oxygen barrier located in this tissue (Perlick and Pühler, 1993). The expression of late-*nodulin* genes is activated around the onset of nitrogen fixation; these gene products are involved in nodule functioning, which is mainly the metabolic exchange between plant and bacteroid (Frühling et al., 1997, Vieweg et al., 2004, Perlick and Pühler, 1993). The most prominent and most thoroughly investigated late *nodulins* are: *leghemoglobins* (Lb)14, *uricase* II, sucrose synthase, and glutamine synthetase (Perlick and Pühler, 1993). These late *nodulins* are important for nodule function and therefore dominate in mature nodules (Perlick and Pühler, 1993). *Leghemoglobins* (Lb) are expressed in the infected cells just before and during nitrogen fixation. These oxygen-binding heme proteins are responsible for supporting and controlling the flux of oxygen to the nitrogen- fixing bacteroids (Vieweg et al., 2004, Perlick and Pühler, 1993). *Uricase* II is the key enzyme of the *ureide* pathway in tropical legumes, where *ureides* are used to translocate the fixed nitrogen to other plant organs. Glutamine synthetase catalyzes the synthesis of glutamine, which is the major transport intermediate for nitrogen in temperate legumes. Sucrose synthase provides for monosaccharides by cleaving sucrose to UDP-glucose and fructose (Perlick and Pühler, 1993). The leghemoglobin gene *VfLb29* from *V. faba* is the first late-nodulin gene which has been shown to be transcriptionally activated not only in the root nodule, but also in roots

colonized by an endomycorrhizal fungus. Interestingly, *VfLb29* exhibits a significantly low sequence homology to other *V. faba* leghemoglobin genes as well as to leghemoglobin genes from other legumes (Vieweg et al., 2004).

3.6 Pesticides

When pesticides are used to fight infection with pests that are pathogens for the plant, most of the chemical treatment will accumulate in the soil (Ahemad and Khan, 2013). These chemical have the potential to reduce the beneficial signaling between bacteria and plant, which affects the formation of nodules, plant growth, and yield (Ahemad and Khan, 2013).

Globally, the use of pesticides on leguminous plants has undergone a remarkable upward push over the last few years (Gupta et al., 2013). Pesticides are defined as: "substances or mixtures of substances intended for preventing, destroying, repelling or mitigating pests, and the major groups of pesticides are fungicides, herbicides, and insecticides" (Newman et al., 2016). In another definition, "pesticides are bioactive, toxic substances, and they influence, directly or indirectly, soil productivity and agroecosystem quality" (Imfeld and Vuilleumier, 2012).

Pesticides include herbicides, insecticides, nematicides, and fungicides and are used in modern agriculture to obtain sensible and low priced food and fiber (Imfeld and Vuilleumier, 2012, Gupta et al., 2013). They are composed of a diverse group of inorganic and organic chemicals pesticides and represent major inputs to agricultural production systems (Imfeld and Vuilleumier, 2012).

Although the use of pesticides has managed to increase agricultural production, they have been linked to several important health and environment related issues (Walvekar et al., 2017, Gupta et al., 2013). Application of pesticides on agricultural crop is now a common practice and is an important factor of integrated pest management (IPM) strategies (Gilani et al., 2010b). Pesticides

are essential to farmers for the prevention of plant pests and illnesses. “It's estimated that about 45% of the world crop is ruined by plant sickness and pests” (Bhanti and Taneja, 2007). Pesticides are broadly used to fight a wide range of pests infesting agricultural crops. Globally, it is assed that about $3x10^9$kg of pesticides are applied annually with a purchase price of nearly $40 billion each year (Hussain et al., 2009). To minimize crop loss, it is necessary to use pesticides for the period of growth, storage, and transport. However, the arbitrary use of pesticides can result in their accumulation within the environment (Singh et al., 2015b).

Accumulation in soil unfavorably affects its properties and changes soil pH that, in turn, affects microbial activities (Gilani et al., 2010b). The uncritical use of pesticides has resulted in heavy pollution of soil and water bodies (Walvekar et al., 2017). It is assessed “that less than 0.03% of all the pesticides affect their targeted pests, and the remaining 99.97% accumulates in the environment “ (Singh et al., 2015b). A study with mirid insects on cocoa estimated that “the percentage was almost the same or 0.02% of the applied insecticide reaching the mirids” (Pimentel and Burgess, 2012, Pimentel, 1995). In another study it was found that approximately less than 0.1% of applied pesticide reaches the target pest, leaving the bulk to affect the environment (Gilani et al., 2010b).

With the rising use of pesticides in current agriculture, the concern of the impact of these chemicals on the structure of soil microorganisms has received more attention (Hussain et al., 2009). The applied pesticides may harm the indigenous microorganisms (Hussain et al., 2009, Singh et al., 2015b), disturb soil ecosystem, and therefore may affect human health by inflowing in the food chain (Hussain et al., 2009). The effects of pesticides on the soil microflora are of significance because many microbial functions are important to crop production, soil fertility, soil sustainability, and environmental quality (Imfeld and Vuilleumier, 2012, Lo, 2010); it may also

influence soil biochemical processes depending on microbial and enzymatic reactions (Hussain et al., 2009).

In soil ecosystems, microbial communities offer important processes like recycling of nutrients and decomposition of natural material; they also represent an important food source at the base of food webs and biogeochemical cycling. Furthermore, microbial metabolism in contaminated soil leads to the removal of organic pollutants in a process known as remediation (Gupta et al., 2013, Wang et al., 2008, Hussain et al., 2009, Imfeld and Vuilleumier, 2012, Lo, 2010, Muñoz-Leoz et al., 2013).

Pesticides in soil go through diverse procesess of degradative, transport, and adsorption /desorption depending on the chemical nature of the pesticide and soil properties (Hussain et al., 2009). The interaction of pesticides with soil organisms and their metabolic activities may alter the biochemical and physiological performance of soil microbes (Hussain et al., 2009, Muñoz-Leoz et al., 2013); however, the relationship between the chemical structure of a pesticide and its effect on the different groups of soil microorganisms is not easy to be predicted. Some pesticides have depressive effects or no effects on microorganisms when applied at normal rates, while others can stimulate the growth of microorganisms, (Lo, 2010, Hussain et al., 2009). It is possible that groups of microorganisms may be outnumbered and removed from competition-based networks. Sometimes, microbial population sare affected initially by pesticide application, but with time (after a period of acclimation) the population simply returns to normal or even increases. This is an indication of changes in microbial catabolic capabilities that may be either due to induced pesticide degradation capabilities or due to a change within the microbial community (Hussain et al., 2009).

The hydrophobic nature of pesticides favors binding to soil particles and to display longer perseverance in the environment. Pesticides that contain hydrophilic groups such as carbamates and organophosphates proved to be equally harmful because of their capability to leak into ground water (Walvekar et al., 2017). As these organic molecules are not completely specific to their target, when their accumulation exceeds a threshold limit, their damaging effects can be detected in the ecosystem (Walvekar et al., 2017).

Pesticides can disturb the soil microbial community by several non-target mechanisms: (a) plant growth promoting properties of rhizobacteria like indole acetic acid production and phosphate solubilization are negatively affected by application of these synthetic compounds (Walvekar et al., 2017); (b) pesticides act as carbon and energy sources for specific microorganisms that thrive and outnumber others in a population, causing disparity in soil microflora (Walvekar et al., 2017); (c) pesticides are known to have toxic effects on nitrogen (N) transformation processes (Walvekar et al., 2017); and (d) inhibition of soil enzymes like ureases, alkaline phosphatases and dehydrogenases, are also known to happen in the presence of these xenobiotic (Walvekar et al., 2017, Hussain et al., 2009). Pesticides bioavailability in soil environment is one of the major causes to the net impact on soil microbes. Desorption and adsorption processes can regulate the bioavailability of a pollutant in soil solution, its concentration, and its bioactivity(Hussain et al., 2009).

An important indicator of microbial activities is microbial biomass, it can provide a direct assessment of the linkage among microbial activities and the nutrient transformations also other ecological processes (Muñoz-Leoz et al., 2013, Hussain et al., 2009). Usually, an increase in respiration indicates the enhanced growth of bacterial population and a decrease in soil respiration reflects the reduction in microbial biomass. Some microbial groups are able to use the applied

pesticide as a source of nutrients and energy to multiply, while the pesticide may be toxic to other organisms (Hussain et al., 2009). Specifically, microbial parameters that offer information about the soil nitrogen cycle have been shown to be very sensitive to the application of pesticide (Muñoz-Leoz et al., 2013).

Ideally, target pests should be the only organisms affected by pesticides. Several studies however indicate that pesticides can have a negative effects on non-target organisms as well as on soil microbial communities (Singh et al., 2015b, Muñoz-Leoz et al., 2013). Pesticides often have slow degradation rates in the soil environment depending on several factors such as soil type, soil physicochemical properties, and pesticide composition and biological properties. Accordingly, repeated application of pesticides can eventually lead to their accumulation at concentrations that can be harmful to soil microorganisms (Muñoz-Leoz et al., 2013).

3.7 Study Area: Palestine

In Palestine, the immoderate uncontrolled use of chemical compounds for pests' control and plant sickness abatement has been a serious issue for land-based food production (Al-Sa' ed et al., 2011). This has caused speculation as to link pesticides used and increased rates of breast cancer in the Gaza Strip, and contamination of cow's milk (Al-Sa' ed et al., 2011). There is a vast trade in illegal pesticides into the Occupied West Bank (Watts et al., 2017). In 2010, the Palestinian Central Bureau of Statistics described the usage of pesticide in the West Bank and Gaza as "excessive reduced soil fertility and water pollution " (Watts et al., 2017). It is noted then that "in the West Bank the annual rate of use of pesticides reached 502.7 tons, consisting of about 123 types, fourteen of which are internationally illegal for health reasons" (Watts et al., 2017). The majority of these pesticides are destined for use in Area C, focused in the agricultural effective zones within

the Jordan valley. It is expected that 50 percent of pesticide used in the country are illegal (Watts et al., 2017). The fate and effect of pesticide utility in application in Palestine remains unknown, because of the lack of studies in this field.

3.8 Pesticides used in the experiment

The Three type of pesticides used in this experiment are: Confidor (Imidacloprid), Bogiron (Difenoconazole,) and Durspan (Chlorpyrifos). Confidor (Imidacloprid) [1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine] (an insecticide) has been commercially introduced to the market in 1991 and has been increasingly used ever since. It is a worldwide used insecticide, used mainly to control sucking insects on crops, (e.g. aphids, leafhoppers, thrips, whiteflies, termites), and parasites (e.g. fleas) of dogs and cats. It is a systemic insecticide used for seed treatment, soil, and foliar applications. Imidacloprid belongs to the group of nicotine-related insecticides referred to as neonicotinoids, which act as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) resulting in the impairment of normal nerve function (Tišler et al., 2009). Bogiron (Difenoconazole)(a fungicide) (1-[2-[2-chloro-4-(4-chlorophenoxy) phenyl]-4-methyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4- triazole) is a systemic sterol demethylation inhibitor used against Ascomycetes, Basidiomycetes and Deuteromycetes. (Muñoz-Leoz et al., 2013). By targeting ergosterol biosynthesis by inhibiting the fungal enzyme sterol-1-4-a-demethylase, it is highly effective against diseases caused by various fungi infecting grain crops, sugar beet, and fruits (Hamada et al., 2011, Thom et al., 1997). Based on its structural and chemical properties, difenoconazole should be expected to be relatively persistent in soils, because of its high affinity to bind with soil organic matter (Thom et al., 1997). Durspan (Chlorpyrifos) [diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate] is a organophosphorus insecticide widely used in agriculture for pest control (Lo, 2010).Chlorpyrifos is metabolically activated by oxidative

desulfuration to chlorpyrifosoxon, which produces neurotoxicity following inhibition of target esterases in the peripheral and central nervous systems (Richardson, 1995).

4. Research Problem:

The misuse of pesticides in agriculture has leads to increased concerns with regards to the non-target effect of these pesticides. The accumulation of pesticide residue may affect non-target organisms including microbial communities in the rhizosphere which, in turn, may affect plant growth. It is therefore important to be able to assess the environmental impact of such random use of pesticides in order to prevent damage and to increase public awareness.

The research question:

Do pesticides have a non-target effect on the cultured rhizosphere bacteria and N_2 fixing bacteria in the rhizosphere of *Vicia faba* plant?

5. Objective:

The study was aimed at evaluating the non-target effects of pesticide on plant growth parameters and on the cultured rhizospheric bacterial communities of *Vicia faba* plants, the study also aims to assess the effect of pesticide on cultured N_2 fixing bacteria.

6. Research Methodology:

Pesticide treatments and their concentrations, Sampling and biometric measurement of plant growth parameters were performed as described by (Singh et al., 2015b) with a few modifications. For pesticide residual concentration GC-MS was used according to Quechers method (González-Curbelo et al., 2015) . Planting and isolation of culturable species from soil was conducted following the procedure of Buyer (Buyer, 1995b). Isolate identification was conducted according to colony morphology, gram staining, cell morphology, and biochemical tests.

6.1 Experimental design

Soil was collected from an area behind the Science Faculty building at Birzeit University that has never been treated with pesticides and that had previously been planted with leguminous plants such as broad beans and lentils. The seeds of broad beans used in this experiment were obtained from Anseme company, (genotype negreta extra early purpule seed). The seeds were treated with the fungicide thiram to prevent fungal diseases in seed. In a completely randomized experiment, 81 pots were set up (9 pesticide treatments× 9 replicates =81pots); in each pot, each pesticide was applied at 3 concentrations (3 pesticides×3different concentration =9 treatments). The pots were placed in the open air without controlling condition.

6.2 Pesticide treatments and their concentrations

Three types of pesticides were used in this experiment: Confidor (imidacloprid), Bogiron (Difenoconazole), and Durspan (Chlorpyrifos) (Table 1). The pesticides were applied to the plantlets after 20 days from planting the seeds according to commercial formulations with the following 3 doses: 0 (control), a low dose corresponding to the recommended dose as per

manufacturer instructions, and a high dose (3 times the low dose). The high dose was applied to assess the potential toxicological impact of the pesticide on soil micro-flora in less frequent but practically encountered situations.

Table 1: The three type of pesticides used to treat the plant of broad bean *Vicia fava*.

مدة الرش	High dose ml\L	Low dose ml\L	الاسم الشائع	الشركة المنتجة Formulator	تركيز المادة الفعالة	صورة المبيد	Brand Name	الأسم التجاري
Once\month	3ml\L	1ml\L	Imidacloprid	Lied Chemical LTD	350g/L	SC	Confidor	كنفيدور (حشري)
2 week	1.5ml\L	0.5mlL	Difenoconazole	Dr. Meron	250 g/L	EC	Bogiron	بوجيرون (فطري)
Once\month	4.5ml\L	1.5ml\L	Chlorpyrifos	Dow Agrosciences	479 g\L	EC	Durspan	دورسبان (حشري)

6.3 Sampling and biometric measurement of plant growth parameters

Samples were taken after 45 and 75 days post-pesticide application. Plants were carefully uprooted, and the rhizospheric soil was sampled. Soil samples were put in two tubes, one for immediate bacterial cultivation stored at 4°C, and the other shock frozen by liquid nitrogen and stored at −20 ◦C. Root length was carefully measured from the point of attachment of the stem base to the tip of the taproot. Shoot length was measured from the stem base to the tip. To measure the composite dry weight, plants were dried in an oven at 60 ◦C for 24 h.

6.4 Residual concentrations of pesticides

Residual concentrations of pesticides were measured through GC–MS after 45, and 75 days post treatment. Extraction of chemical pesticides from soil samples was performed according the Quechers procedure**,** from each replicate 3 samples were analyzed:

6.4.1 Sample pretreatment

Soil samples were dried at 50°C overnight. Dried samples were sieved through 435 micrometer sieve, and processed according to the Quechers method (Extraction, and cleanup).

6.4.2 Quechersprocedure

6.4.2.1 Sample Extraction

Three g of air-dried soil sample was weighed (≥70% H_2O content) into a 50mL centrifuge tube and 7mL H_2O were added, vortexed briefly, and allowed to hydrate for 30 min. Ten mL of acetonitrile was added to each sample. Samples were shaken in a Spex Sample Prep Geno/Grinder 2010 operated at 1500 rpm. The contents of an ECQUEU750CT-MPMylar pouch (citrate buffered salts) was added to each centrifuge tube. Samples were immediately shaken for at least 2 min. and then centrifuged for 5 min at ≥3000 rcf.

6.4.2.2 Sample Cleanup

A 1 mL aliquot of supernatant was transfered to a 2mL CUMPSC18CT dSPE tube (MgSO4, PSA & C18). Samples were vortexed for 0.5 - 1 min, then centrifuged for 2 min at high rcf≥5000. The supernatant was filter purified through a 0.2 µm syringe filter directly into a sample vial. Samples treated with Bogiron (Difenoconazole) and Durspan (Chorphrifos) were

analyzed by GC/MS Model (QP-5000 Shimadzu), -GC model (17A), -Autosampler (AOC 17). Samples treated with Confidor (Imidacloprid) were analyzed byHPLC (Alliance, waters 2690 Separation Module with Waters 996 Photo Diode Array Detecter)

6.5 Rhizosphere soil pH determination at the two-time points

The pH of the soil after the two-time sample collection was determined by drying the soil in an oven at 50 ◦C for 30-60 min. 4 ml of distilled water were added to two grams of soil, followed by shaking with a reciprocator machine for 20 minutes. A pH meter was used to measure the pH.

6.6 Planting and isolation of culturable species from soil:

Rhizosphere isolation medium (RIM) has been developed for the enumeration and isolation of soil and rhizosphere microorganisms (Buyer, 1995a). This medium contains glucose and 15 of the 20 common amino acids. The absence of five other amino acids, namely: aspartic acid, asparagine, cysteine, proline, and threonine, inhibits the growth of *Bacillus mycoides*, a commonly encountered bacterium that rapidly spreads on agar media and complicates the isolation and enumeration of other microorganisms. The other medium that was used is Dilute RSM (dRSM). This medium is similar to RIM but it contains the 5 amino acids missing in RIM, both media were prepared to culture the bacteria from the rhizospheric soil. Burk's N-free medium was used to isolate N_2 fixing bacteria.

I- Rhizosphere isolation medium (RIM) was prepared as follows: 10.0 ml of 100x $Ca(NO_3)_2$, 1.0 ml of 1 M $MgSO_4$, 9.11 g of ACES (*N*-[2-acetamido]-2-amino-ethanesulfonic acid) (Sigma), and 1.0 g of NaOH were dissolved in 500 ml of H_2O (all stock solutions are given in Table 2). pH was adjusted to be 6.7 - 6.9, 15 g of agarose (Life Gene) was added. The mixture was brought to 1,000 ml with H_2O and autoclaved (Tuttnauer autoclave -stem sterilizer, model (3870M)) at 121°C for 30 min. After cooling to 45 to 50 °C, the following sterile stock solutions were added: 1.0 ml of 1 M KH_2PO_4, 1.0 ml of glucose, 1.0 ml of amino acid mixture, 1.0 ml of tyrosine, 1.0 ml of vitamins (Table 2), 20.0 ml of cycloheximide, and 5.0 ml of nystatin(Sigma). Plates were poured, allowed to set at room temperature, and stored at 4 °C until using.

II- Dilute RSM (dRSM) was prepared identically, except that sucrose was used instead of glucose and 1.0 ml of amino acid mixture (containing tryptophan, cysteine, proline, aspartic acid, and asparagine) was used instead of the amino acid mixture of RIM and tyrosine. 40 g of Trypticase soy agar (Sigma) was added instead of agarose. ACES-buffered saline contained 8.5 g of NaCl and 3.6 g of ACES per liter and was adjusted to pH 6.7 to 6.9 with NaOH before autoclaving.

III- The nitrogen-fixing media (Burk's N-free medium) contained the following ingredients: sucrose, 20.0 g; Magnesium sulphate 0.200g, Dipotassium phosphate 0.800g, Monopotassium phosphate 0.200g, Calcium sulphate 0.130g Ferric sulphate 0.00145g, molybdate 0.000253g nd agar, 15 g. The pH was adjusted to 7.3 and autoclaved at 121 ºC for 30 min.

Table 2: Stock solution used in the preparation of RIM and dRSM media (Buyer, 1995b)

Stock	Ingredient	Concn (g liter^{-1})	Diluent	Treatment
100× $Ca(NO_3)_2$	$Ca(NO_3)_2 \cdot 4H_2O$	75.1	H_2O	Autoclave
1 M $MgSO_4$	$MgSO_4 \cdot 7H_2O$	246.5	H_2O	Autoclave
1 M KH_2PO_4	KH_2PO_4	136.1	H_2O	pH 7 with NaOH, autoclaving
Glucose	Glucose	100.0	H_2O	Autoclave
Amino acid mixture	Gly, Ala, Val, Leu, Ile, Ser, Met, Phe, Trp, Glu, Gln, His, Arg, Lys	6.7 of each amino acid	H_2O	Autoclave
Tyrosine	Tyr	6.7	H_2O	NaOH to dissolve, autoclaving
Vitamin	Biotin	0.10	H_2O	Filter sterilization
	Niacinamide	0.35		
	Thiamine · 2HCl	0.30		
Cycloheximide	Cycloheximide	20.0	H_2O	Filter sterilization
Nystatin	Nystatin	10.0	Methanol	

6.6.1 Extraction of bacteria from soil:

Bacteria were extracted from soil and rhizosphere samples by placing approximately 1 g of soil or roots plus adhering soil into a sterile Erlenmeyer flask. A volume of ACES-buffered saline (0.1 M) equivalent to nine times the sample weight was added, then the flask was shaken at 200 rpm for 10 min. The slurry was serially diluted 10-fold in sterile ACES-buffered saline.

ACES-buffered saline (0.1 M) was prepared by adding 18.22g of ACES to 750 ml of dH_2O, pH was adjusted (6.1 -7.2) by adding NaOH, the mixture brought to 1,000 ml with H_2O and autoclaved (tuttnauer autoclave -stem sterilizer, model (3870M)) at 121 °C for 30 min.

6.6.2 Enumerations: The diluted extracts (10^4, 10^5 and 10^6) were plated in duplicate. After incubation at 28°C for 2 to 3 days, the colonies were counted manually by using acolony counter (model No: cc-1).

6.6.3 Identification of bacteria: Single colonies from the both media (RIM and dRSM) were isolated and analyzed by streaking on Trypticase soy agar (Difco), incubated at 28°C for 24-72 hr., the colonies were re-streaked to ensure purity.

6.6.4 Characterization of the isolates

Preliminary characterization was performed using morphological and cultural characteristics. Morphological identification of the isolates was done under the dissecting and compound microscope to observe cell size, shape and arrangement characteristics after classical staining of bacteria. Biochemical tests included; citrate utilization, gelatine liquefaction, methyl red-Voges Proskauer, urease test, nitrate reduction test, motility, indole production test, H_2S production, catalase test, starch hydrolysis, oxidase test, phosphate solubilization test (Pikovskaya agar, Himedia), and resistance to Amoxicillin and Vancomycin.

6.7 Statistical analysis

All of the data were expressed as means ± standard deviation (SD). Data were analyzed by one - way ANOVA using Statistical Package for Social Studies (SPSS) software (version 20). This test was performed to assess the effect of pesticide type and application rate on treated and untreated soil samples, plant growth parameters, pesticide residue, soil pH and bacterial count (CFU g−1 soil) at both dosage and at both time points. One-way ANOVA: post Hoc multiple comparisons Tukey test at significant level of $p \leq 0.05$ was used to compare means within treatments. Student t-test was used to determine significant differences in pH at both time points where values of $p < 0.05$ were considered significantly different.

7. Results and discussion

Plants undergo various stages of growth and development that lead to continuous changes of rhizospheric microbial community structure and function (Walvekar et al., 2017), these changes could also occur if plants are subjected to different stressors such as pesticide exposure, where soil microorganisms are a sensitive indicator of changes in the system (Hussain et al., 2009). The impacts of pesticides on plant growth parameters were checked because these parameters are related to plant health, directly influencing agricultural productivity. In this study three different pesticides (Confidor (Imidacloprid), Bogiron (Difenoconazole), and Durspan (Chlorpyrifos)) were studied for their non-target effect on cultured rhizospheric microbial community structure and function in *Vicia faba*, at two time points (45 days and 75days post-treatment) and two dosages (low dose and high dose). The technique of enumeration of specific groups of rhizospheric microbes was employed to assess microbial community structure.

7.1 Effect of pesticides on plant growth parameters

Confidor and, Bogiron, have no effect on plant leaves and roots health of *Vicia faba* after 75 days of pesticide application (Figure 1, 2 and 3) at both doses. The application of Durspan affects the plant health as shown in Figure 1 (F, G) at both doses burned the leaves causing as the appearance of brown dots as shown in Figure2 (F, G); leaves of plants treated with the higher dose have more brown dots compared to leaves treated with the lower dose. The roots of plants after the application of Durspan become thicker than the control in both doses (Figure3 F, G) after 75 day of pesticide treatment.

Figure 1: Broad bean (*Vicia faba*,) plant comparison A) Control, B) Confidor low dose, C) Confidor high dose D) Bogiron low dose, E) Bogiron high dose, F) Durspan low dose, G) Durspan high dose. Phenotype comparison

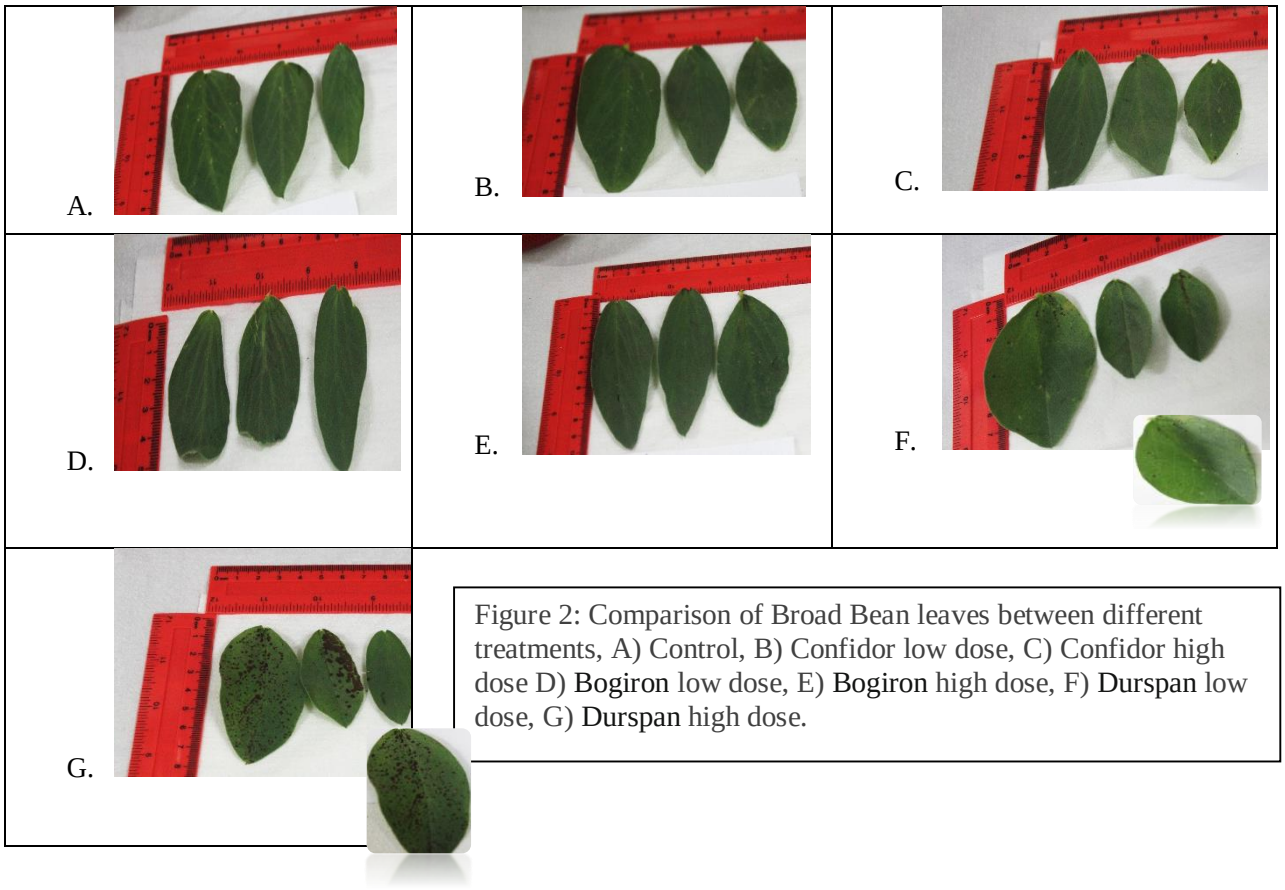

Figure 2: Comparison of Broad Bean leaves between different treatments, A) Control, B) Confidor low dose, C) Confidor high dose D) Bogiron low dose, E) Bogiron high dose, F) Durspan low dose, G) Durspan high dose.

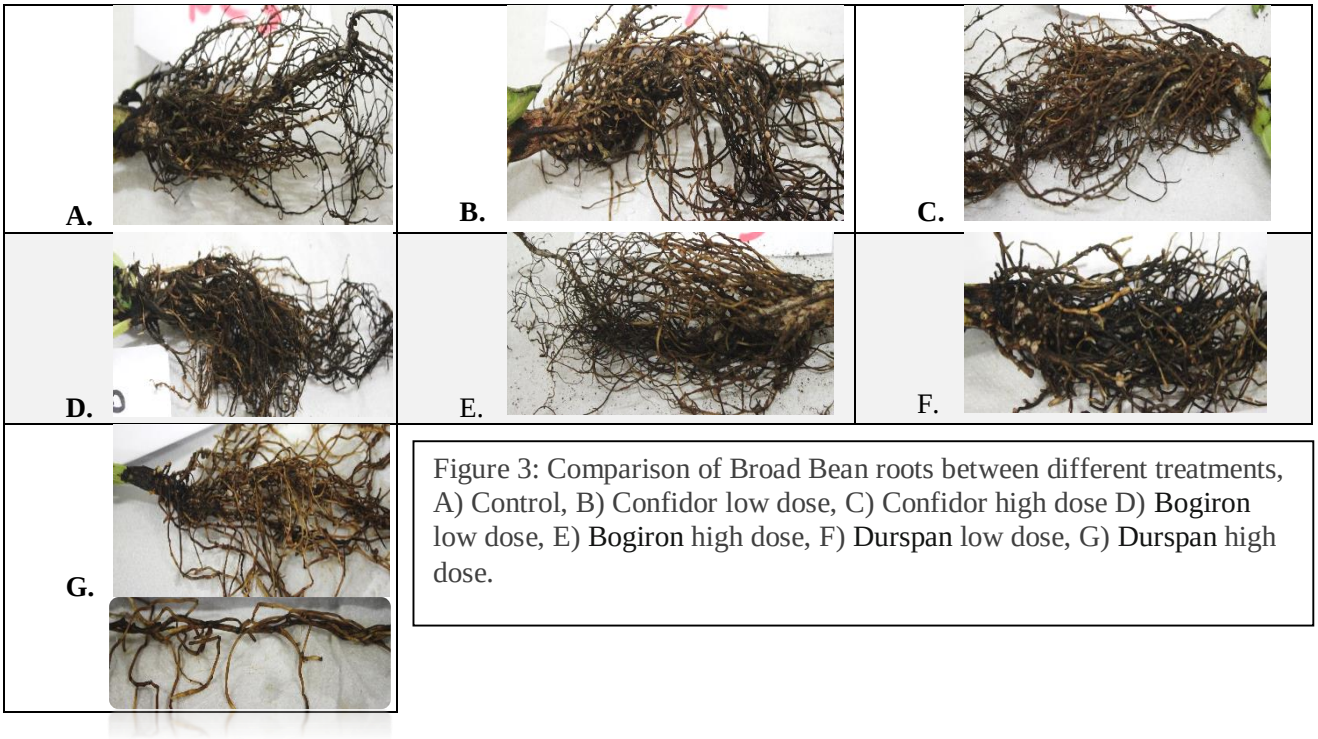

Figure 3: Comparison of Broad Bean roots between different treatments, A) Control, B) Confidor low dose, C) Confidor high dose D) Bogiron low dose, E) Bogiron high dose, F) Durspan low dose, G) Durspan high dose.

Biometric measurements were taken at the two-time points, and one-way ANOVA test was performed to assess significant changes between treatments compared to control. Table 3 shows that the 3 pesticides had no effect on root and shoot length compared to the control 45 days' post treatment. At 75 days' post treatment there was a significant difference between the collected data. One-way ANOVA: post Hoc multiple comparisons Tukey test was done to establish whether there were significant changes between treatments (Table A1 see Annex). For both Confidor and Bogiron at 75 days' post treatment there was no significant difference compared to the control (all measurements Table 3), but for Durspan there was a significant difference compared to control in the root length ($p=0.031$). At 45-day post treatment there was no effect of Confidor and Bogiron on dry matter compared with the control (Table3).

The impact of the pesticides on plant growth parameters was examined because these parameters are related to plant health, that is directly affecting agricultural productivity (Singh et al., 2015b). In the present study Confidor and Bogiron had no effect on plant health compared with the control, whereas Durspan affected plant roots and leaves also the root length was decreased. A similar study but on a different plant done by (Singh et al., 2015b) on *Vigna radiata* showed that high dose of chlorpyrifos increased plant dry weight. Another study done by (Walvekar et al., 2017) also on *Vigna radiata* showed that chlorpyrifos positively affected shoot length, and increased the dry weight. These changes may occur after the application of pesticide because of the plant need to adapt to environmental change and stress (Baligar et al., 2001). Thickening of roots after application of Durspan could be the result of the plant need to uptake more nutrient from soil to enhance the health and survival of the plant (Baligar et al., 2001). The root morphological factors for example length, thickness, surface area, and volume have intense effects on the ability of the plant to obtain and absorb nutrients from soil. While these parameters derived the capability of the

roots to reach a high bulk of the soil layers, to tolerate moisture extremes and temperature also tolerate deficiencies of elements and toxicities (Baligar et al., 2001).In a previous study, the effect of Durspan has been studied on root-mitosis of *Vicia faba*. The results induced a significant percentage of abnormal mitoses, the percentage of which increased as the concentration of the experimental agent increased. The different treatments with Dursban did not affect the mitotic index. Disturbed meta and anaphases where the chromosomes spread irregularly over the cell dominated. It also induced a considerable percentage of lagging chromosomes and chromosome fragmentation which may induce micronuclei (Amer and Farah, 1983).

Table 3: Broad bean plant parameters under different pesticide treatments at 45 and 75 days' post treatment (4 replicate for each treatment)

Treatments 45 day		**Root L (cm)**	**Shoot L (cm)**	**Dry matter (g)**	**Treatments 75 day**		**Root L (cm)**	**Shoot L (cm)**	**Dry matter (g)**
C45	Mean	56.67	29.00	5.00	C75	Mean	50.00	85.00	12.50
	Std. Deviation	28.290	3.464	1.000		Std. Deviation	10.000	18.193	1.572
CL45	Mean	73.33	29.33	5.33	CL75	Mean	43.67	67.00	13.50
	Std. Deviation	14.364	6.351	2.887		Std. Deviation	2.082	6.083	1.375
CH45	Mean	52.00	28.00	5.00	CH75	Mean	43.67	88.00	15.27
	Std. Deviation	6.083	6.928	2.646		Std. Deviation	2.082	15.716	3.066
BL45	Mean	29.67	32.33	5.33	BL75	Mean	42.00	60.00	11.30
	Std. Deviation	14.844	4.041	2.082		Std. Deviation	4.583	20.000	2.464
BH45	Mean	73.33	34.67	6.33	BH75	Mean	46.00	76.67	13.60
	Std. Deviation	6.807	6.658	1.528		Std. Deviation	5.292	13.013	2.629
DL45	Mean	80.00	29.67	6.00	DL75	Mean	41.00	59.67	8.23
	Std. Deviation	13.000	.577	1.000		Std. Deviation	10.149	16.197	5.953
DH45	Mean	72.33	29.00	5.33	DH75	Mean	**31.33**	65.33	6.70
	Std. Deviation	32.347	4.000	1.155		Std. Deviation	3.215	8.083	1.916
p-value		0.068	0.686	0.967			0.155	0.069	0.040

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

7.2 Pesticide concentrations in the soil

The residual concentrations of Confidor, Bogiron, and Durspan are shown in Table 4. After 45 days at low dose the residual concentrations were 6 mg/kg soil, 7 mg/kg soil, and 20.66 mg/kg soil, respectively. At high dose the residual concentrations were was 24 mg/kg soil, 25.6 mg/kg soil, and 72.3 mg/kg soil, respectively. After 75 days at low dose the residual concentrations were 8.66 mg/kg soil, 6 mg/kg soil, and 4.66 mg/kg soil, respectively. Whereas at high doses it was 18.33 mg/kg soil, 55.33 mg/kg soil, and 13.33 mg/kg soil, respectively. Note that the application of pesticide was according to the manufacturer recommended dose and to mimic what Palestinian farmers use in the field (Table1). The three pesticides were applied as recommended by the manufacturers: Confidor and Durspan once a month, Bogiron twice a month.

Table 4: Pesticide residual concentration in soil after 45 and 75 day of treatment (3 replicate for each treatment)

Pesticide concentration 45 day mg/kg			**Pesticide concentration 75 day mg/kg**		
Treatments	Mean	Std. Deviation	Treatments	Mean	Std. Deviation
CL45	6.0	2	CL75	8.7	3
CH45	24.0	7	CH75	18.3	9
BL45	7.0	3	BL75	6.0	1
BH45	25.7	5	BH75	55.3	16
DL45	20.7	13	DL75	4.7	3
DH45	72.3	33	DH75	13.3	9
p-value	<0.001		p-value	<0.001	

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Figure 4 represents the pesticide residue concentration in soil between the two time points and both pesticide doses, ANOVA test results show a significant difference in the residual concentration. The one-way ANOVA: post Hoc multiple comparisons Tukey test was done to assess the

significant changes between treatments (Table A2 see Annex). There was a significant difference in the residual concentration of Bogiron between the low dose and high dose after 75 day of the treatments (p=.003). Significant differences were also recorded with Durspan between low dose and high dose after 45 day of the treatments (p=.002), and between the Durspan high dose at both time points (p<0.001).

The three pesticides studied differed widely in their degradation behavior. Bogiron shows a slower degradation than Confidor and Durspan. For Durspan there was a significant decrease in the residual concentration after 75 day for both doses, which is in accordance with the study by Singh and Sharma that shows Chloropyrifos almost complete degradation toward the end of the experiment (80 days) (Singh et al., 2015b). This result is in agreement with the findings of Gilani and colleagues (Gilani et al., 2010b): Chlorpyrifos is moderately persistent in soils with half-life usually between 60 and 120 days, but can range from 2 weeks to over 1 year, depending on the soil type, climate, and other conditions. Imidacloprid shows a faster degradation in soil for both doses; on the other hand, a study by(Cycoń et al., 2013) showed that degradation of imidacloprid in non-sterile soil with naturally occurring microorganisms was slow. However, the degradation rate of the insecticide was dependent on the used concentration. Difenoconazole shows that the degradation rate slows down with time, which is consistent with a similar study by (Banerjee et al., 2008) that shows that the dissipation rate of Difenoconazole was faster at the beginning and slowed down with the passage of time.

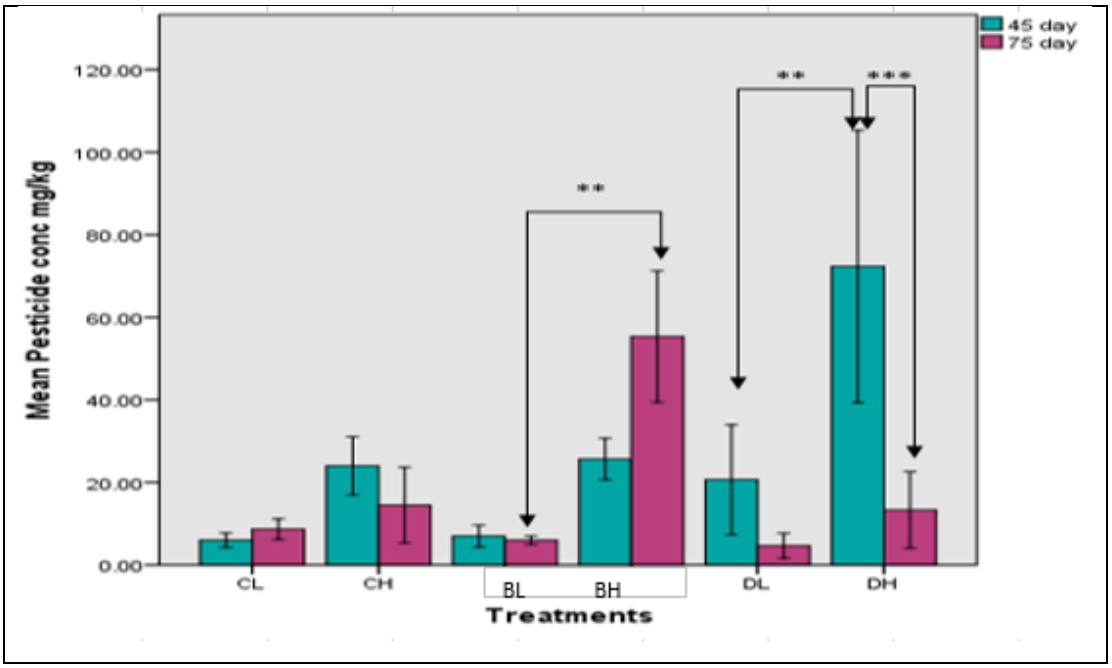

Figure 4: Pesticide residual concentration in soil after 45 and 75-day post pesticides treatment. CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) among the two-time points, and differences between both doses are marked by *. Error bars represent standard deviations.

7.3 Rhizosphere soil pH determination at the two-time points

Rhizosphere soil pH was measured after collection at 45 and 75 days (Table 5). There was no significant effect of the application of pesticide compared with the control on the pH of the rhizospheric soil. One-Sample T-Test was done (Table A3 see Annex) to compare pH at two time points and it shows a significant decrease of the pH at 75 days compared with 45 days ($p<0.001$). However, the decrease of the rhizosphere soil pH is likely caused by roots through soil acidification (Schaller, 1987). Growth of the plant and nutrient uptake result in some localized acidification around plant roots through the exudation of acids from the roots (Goulding, 2016). Also plant roots lower the pH of the rhizosphere if they take up more cations than anions, and the extent of this acidification depends on the rate of proton release by the roots and their diffusion into the soil (Schaller, 1987).

Table 5: Rhizosphere soil pH at the two-time points of post pesticide treatments(3 replicate for each treatment)

pH 45 day			**pH 75 day**		
Treatments	Mean	Std. Deviation	Treatments	Mean	Std. Deviation
C45	7.87	0.15	C75	7.07	0.42
CL45	7.83	0.06	CL75	7.13	0.04
CH45	7.87	0.06	CH75	7.17	0.06
BL45	7.83	0.04	BL75	7.17	0.06
BH45	7.83	0.10	BH75	7.10	0.14
DL45	7.9	0.10	DL75	7.17	0.15
DH45	7.93	0.04	DH75	7.30	0.10
p-value	0.834			0.837	

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

7.4 Effect of pesticide on rhizospheric microbial community structure

Two different media were prepared to assess the effect of pesticides on the cultured rhizospheric microbial community, the selected medium would have a great impact on both total count and the relative abundances of numerous taxonomic groups of microorganisms (Buyer, 1995a). Plating soil bacteria on different media was found to be significantly different from each other depending on the media nutrients and amino acids (Sørheim et al., 1989).

The colony forming units (CFUs) were counted on both media (RIM and dRSM) at two-time points, after 45 days the extract dilution which gave countable colonies on the plates was 10^4, but after 75 days it was 10^5. Table 6 shows CFUs values on both media; the colony type on both media was assessed according to differences in their morphology. The cultured rhizosphere bacteria on both media (RIM and dRSM) at both time point of post pesticide treatment are shown in Figure 5 and Figure 6 respectively.

The data from both media show that there was a significant difference in the CFU between treatment showed in table 6. One-way ANOVA: post Hoc multiple comparisons Tukey test ((Table

A4 see Annex) was carried out to identify significant differences between the treatments compared with the control, differences between both doses and the differences between both time points. Figure7.I shows differences in CFU count for the RIM medium at both time points. At the first time point there was a significant decrease in the CFU count between the Confidor low dose (p=0.012), Bogiron high dose (p=0.016), Durspan low dose (p=0.008) and Durspan high dose (p=0.019) compared with the control 45 days post treatment, there was also a significant difference between both doses of Confidor (p=0.015) where the high dose has a higher number of CFU compared to the low dose.

Table 6: cultured rhizosphere bacteria on RIM and dRSM media: colony forming unit count at both time points of post pesticide treatment (3 replicate for each treatment and was plated in duplicate).

Treatments 45 day		**#CFU\ml-RIM**	**#CFU\ml-DRSM**	**Treatments 75 day**		**#CFU\ml-RIM**	**#CFU\ml-DRSM**
C45	Mean	3.70E+07	4.45E+07	C75	Mean	1.49E+07	1.68E+08
	Std. Deviation	4.24E+06	7.78E+06		Std. Deviation	3.04E+06	1.20E+07
CL45	Mean	9.80E+06	1.11E+07	CL75	Mean	3.25E+08	2.41E+08
	Std. Deviation	1.10E+07	2.12E+05		Std. Deviation	3.46E+07	4.81E+07
CH45	Mean	3.58E+07	1.70E+07	CH75	Mean	1.80E+07	1.60E+08
	Std. Deviation	1.13E+06	2.62E+06		Std. Deviation	2.19E+06	2.40E+07
BL45	Mean	2.05E+07	1.20E+07	BL75	Mean	6.10E+08	9.35E+07
	Std. Deviation	7.07E+05	3.11E+06		Std. Deviation	1.41E+07	7.07E+05
BH45	Mean	1.11E+07	5.40E+06	BH75	Mean	1.43E+08	9.55E+07
	Std. Deviation	6.08E+06	1.70E+06		Std. Deviation	6.08E+07	3.54E+06
DL45	Mean	7.75E+06	8.00E+06	DL75	Mean	3.10E+07	9.00E+07
	Std. Deviation	7.07E+04	1.41E+06		Std. Deviation	1.70E+07	5.66E+06
DH45	Mean	1.21E+07	1.26E+07	DH75	Mean	1.65E+07	3.70E+07
	Std. Deviation	3.04E+06	1.63E+06		Std. Deviation	2.12E+06	8.49E+06
p-value		0.002	<0.001	p-value		<0.001	<0.001

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

75 days post treatment on RIM media: Confidor low dose ($p<0.001$), Bogiron low dose ($p<0.001$), and Bogiron high dose ($p=0.024$), show a significant increase in the CFU count compared with the control (Figure 7.II). The low dose of Confidor ($p<0.001$), and Bogiron ($p<0.001$), show a significant increase of CFU numbers compared with the higher dose. Figure 8. I show CFU count on dRSM medium at both time points. At the first time point there was a significant decrease in the CFU for all treatments compared with the control: Confidor low dose ($p<0.001$), Confidor high dose ($p=0.001$), Bogiron low dose ($p<0.001$), Bogiron high dose ($p<0.001$), Durspan low dose ($p<0.001$), Durspan high dose ($p<0.001$). At the second time point on dRSM media there was a significant decrease in CFU for Durspan high dose ($p=0.005$), compared with the control (Figure 8. II).

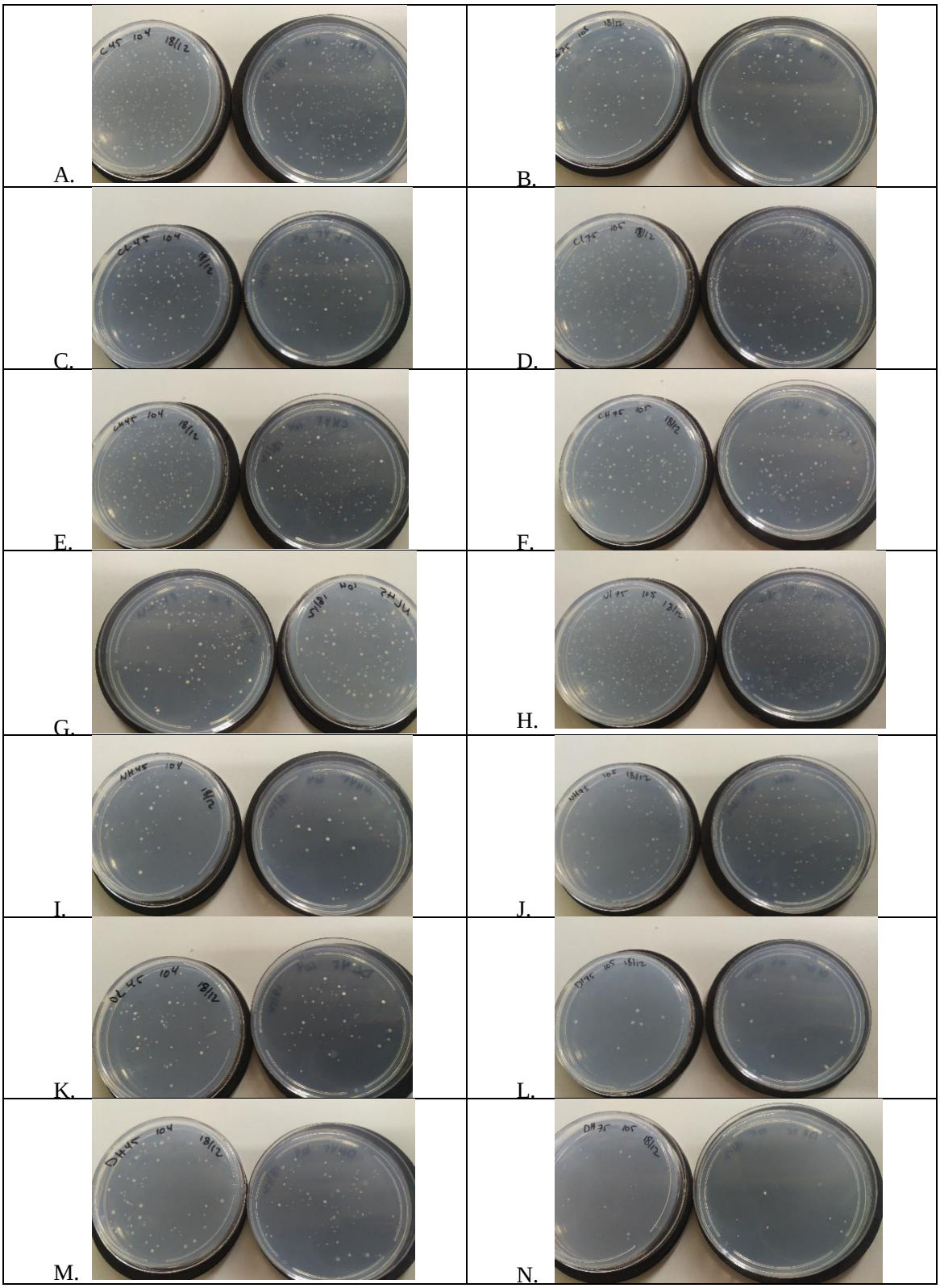

Figure 5: cultured rhizosphere bacteria on RIM media on both time point of post pesticide treatment. A) C45, B) C75, C) CL45, D) CL75, E) CH45, F) CH75, G) BL45, H) BL75, I) NH45, J) NH75, K) DL45, L) DL7, M) DH45, N) DH75.C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

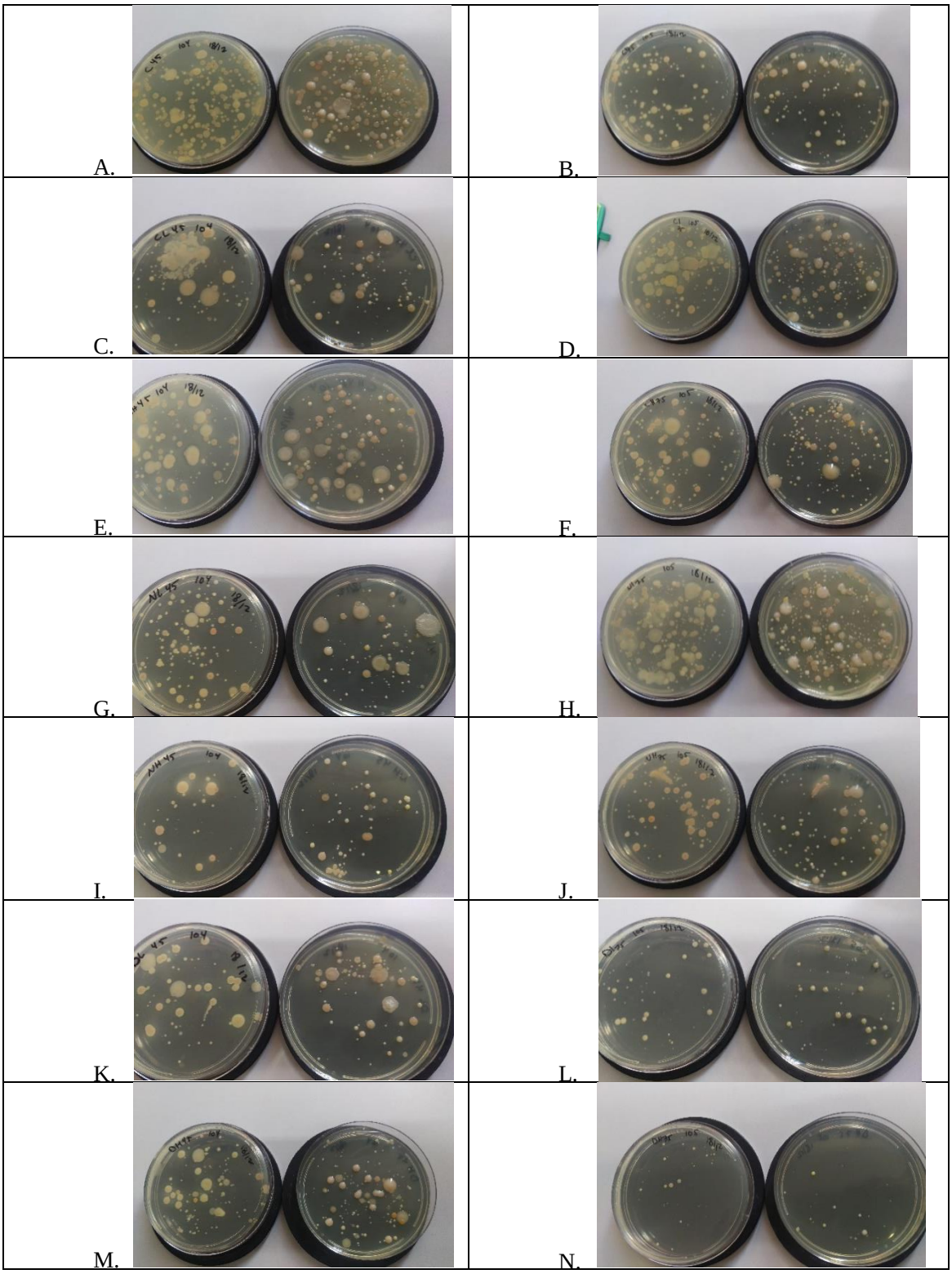

Figure 6: cultured rhizosphere bacteria on dRSM media on both time point of post pesticide treatment .A) C45, B) C75, C) CL45, D) CL75, E) CH45, F) CH75, G) BL45, H) BL75, I) BH45, J) BH75, K) DL45, L) DL7, M) DH45, N) DH75. C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

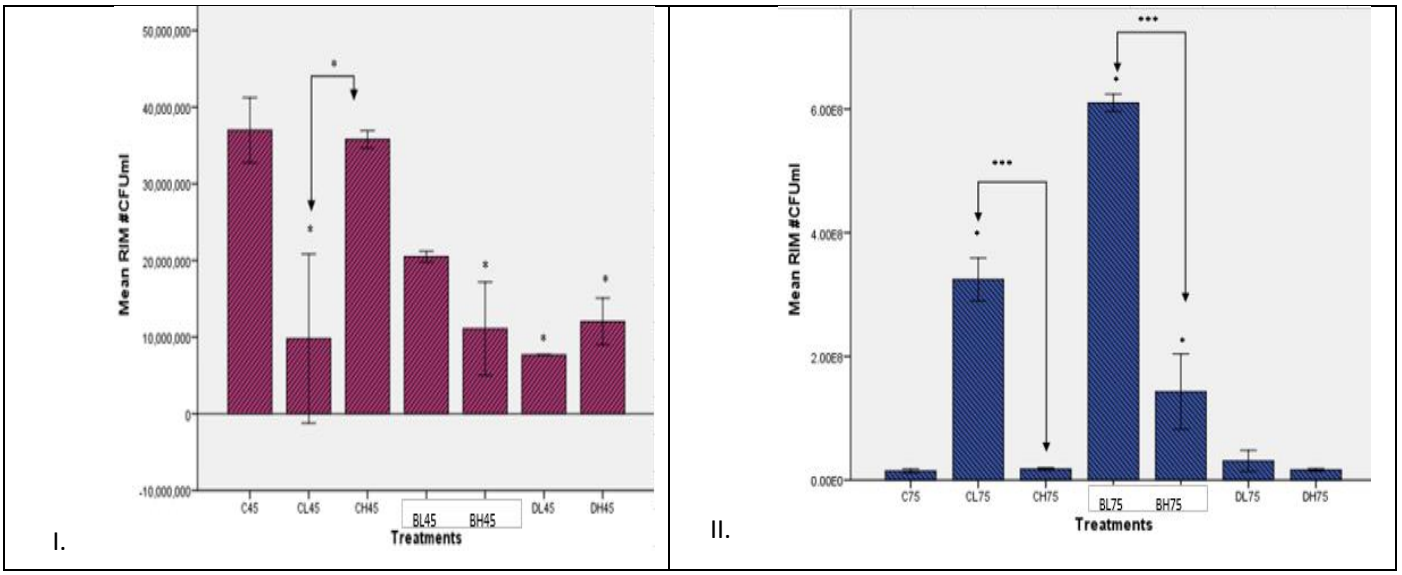

Figure 7: Difference in CFU of cultured rhizosphere bacteria on RIM media, I) 45 day, II) 75 day. C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) between the control and the treatments are representing by *.

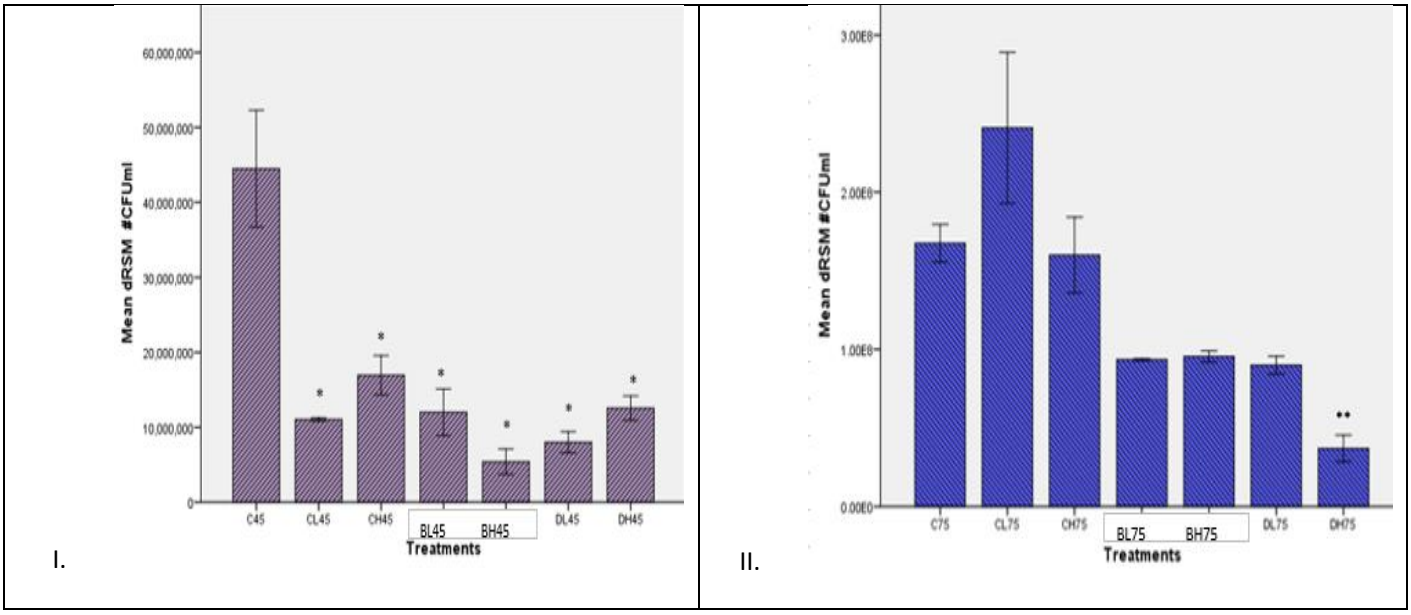

Figure 8: Difference in CFU of cultured rhizosphere bacteria on dRSM media, I) 45 day, II) 75 day of post pesticide treatment. C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) between the control and the treatments are representing by *. Error bars represent standard deviations

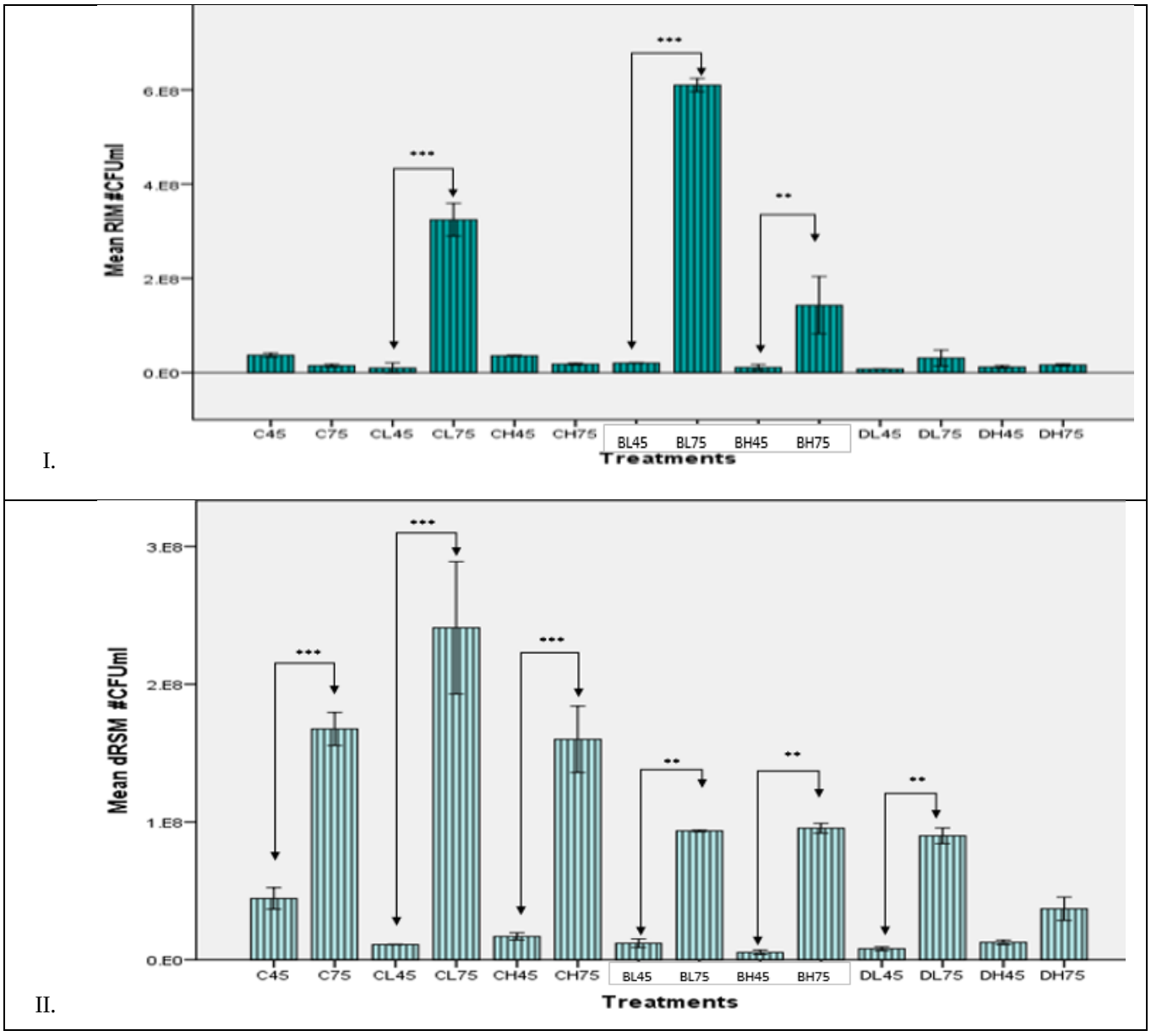

Figure 9: Difference in CFU of cultured rhizosphere bacteria on I.RIM media and II. dRSM media between both time point of post pesticide treatment C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) at two-time point for each treatment are representing by *. Error bars represent standard deviations

Figure 9. I and II represent the difference in CFU of cultured rhizosphere bacteria on RIM media and dRSM media between both time point for each treatment. For RIM media (Figure 9. I), there was a significant increase in CFU counts for Confidor low dose ($p<0.001$), Bogiron low dose ($p<0.001$), and Bogiron high dose ($p=0.001$) at the second time point compared with the first time point. For dRSM media (Figure 9. II) there was a significant increase in CFU count for all the treatments at the second time point (Control ($p<0.001$), Confidor low dose ($p<0.001$), Confidor high dose ($p<0.001$), Bogiron low dose ($p=0.005$), Bogiron high dose ($p=0.002$), and Durspan low dose ($p=0.004$)) compared with the first time point.

The average CFU count from both media was measured to assess the effect of the 3 pesticides on the total cultured bacteria (Table 7), ANOVA test show there was a significant difference in CFU count between the treatments. One-way ANOVA: post Hoc multiple comparisons Tukey test result was carried out (Table A5 see Annex) to identify significant differences between treatments compared with the control, the significant differences between both doses, and significant differences between both time points.

Table 7: Average CFU of cultured rhizosphere bacteria on both media (RIM and dRSM) at both time point post pesticide treatment

CFU after 45 day			**CFU after 75 day**		
Treatments	Mean	Std. Deviation	Treatments	Mean	Std. Deviation
C45	4.08E+07	6.70E+06	C75	9.12E+07	8.84E+07
CL45	1.04E+07	6.41E+06	CL75	2.83E+08	5.91E+07
CH45	2.64E+07	1.10E+07	CH75	8.90E+07	8.32E+07
BL45	1.63E+07	5.24E+06	BL75	3.52E+08	2.98E+08
BH45	8.25E+06	4.91E+06	BH75	1.19E+08	4.46E+07
DL45	7.85E+06	8.35E+05	DL75	6.05E+07	3.56E+07
DH45	1.23E+07	2.01E+06	DH75	2.68E+07	1.29E+07
p-value	<0.001		p-value	0.010	

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Figure 10.I represent the average CFU count at the first time point, it shows that the application of pesticides for both doses significantly decreases the CFU (Confidor low dose ($p<0.001$), Confidor

high dose ($p=0.044$), Bogiron low dose ($p<0.001$), Bogiron high dose ($p<0.001$), Durspan low dose ($p<0.001$), Durspan high dose ($p<0.001$) when compared to the control, also there was a significant difference between both doses of Confidor, where the high dose shows higher CFU count compared with the low dose. For the second time point (Figure 10. II), the decrease in the CFU count at the first time point is no longer present for all the treatments.

Figure 11 represents the difference between CFU count for each treatment between both time points. Confidor low dose ($p=0.006$) and Bogiron low dose ($p<0.001$) show a significant increase in the CFU at the second time point compared to the first time point. This data show that the application of low dose of Confidor and Bogiron could enhance the growth of cultured rhizospheric bacteria with time

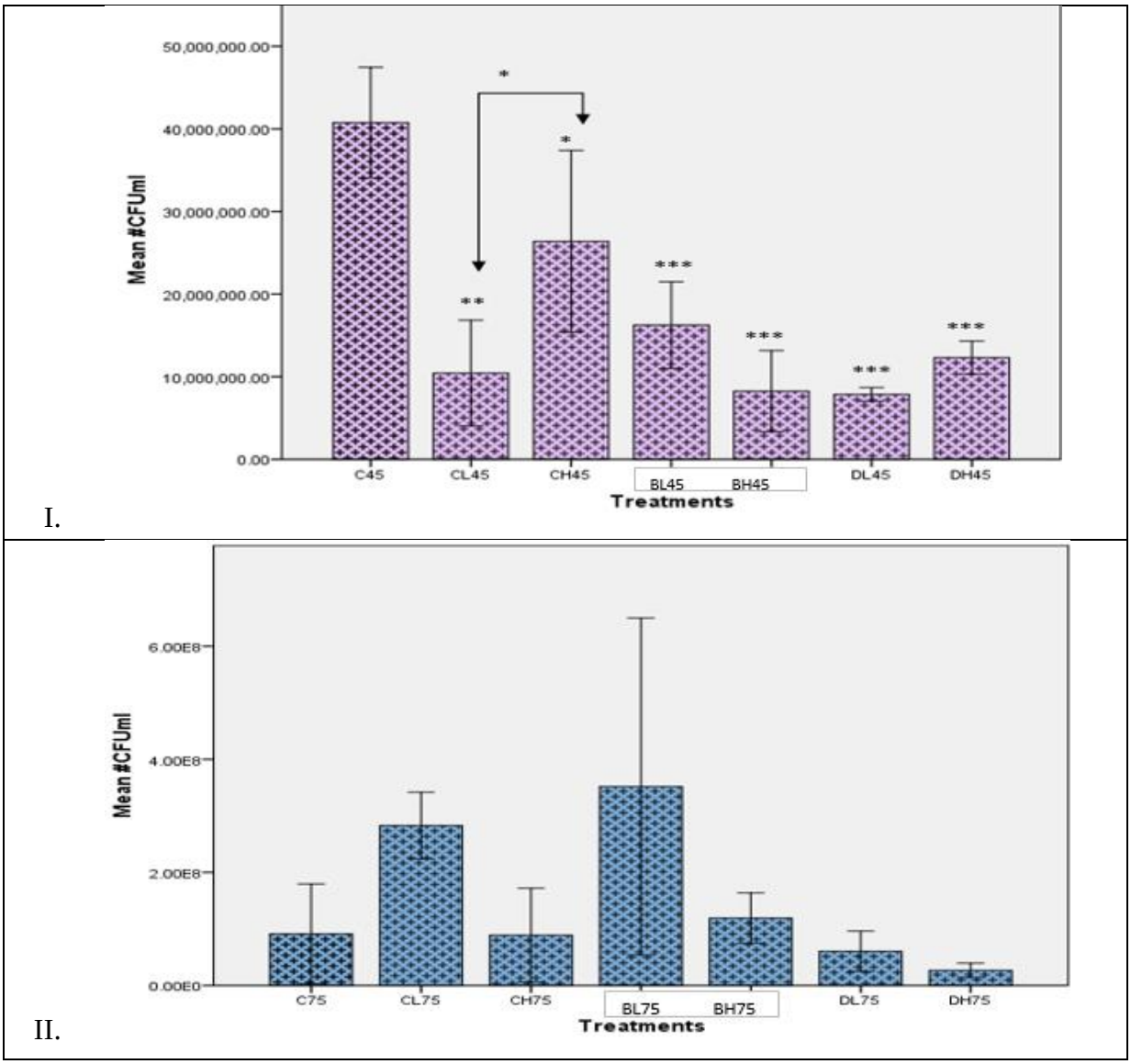

Figure 10: average CFU of cultured rhizosphere bacteria from both media (RIM and dRSM) I) 45 days post treatment, II) 75 days post treatment. C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) between the control and the treatments are represented by *.

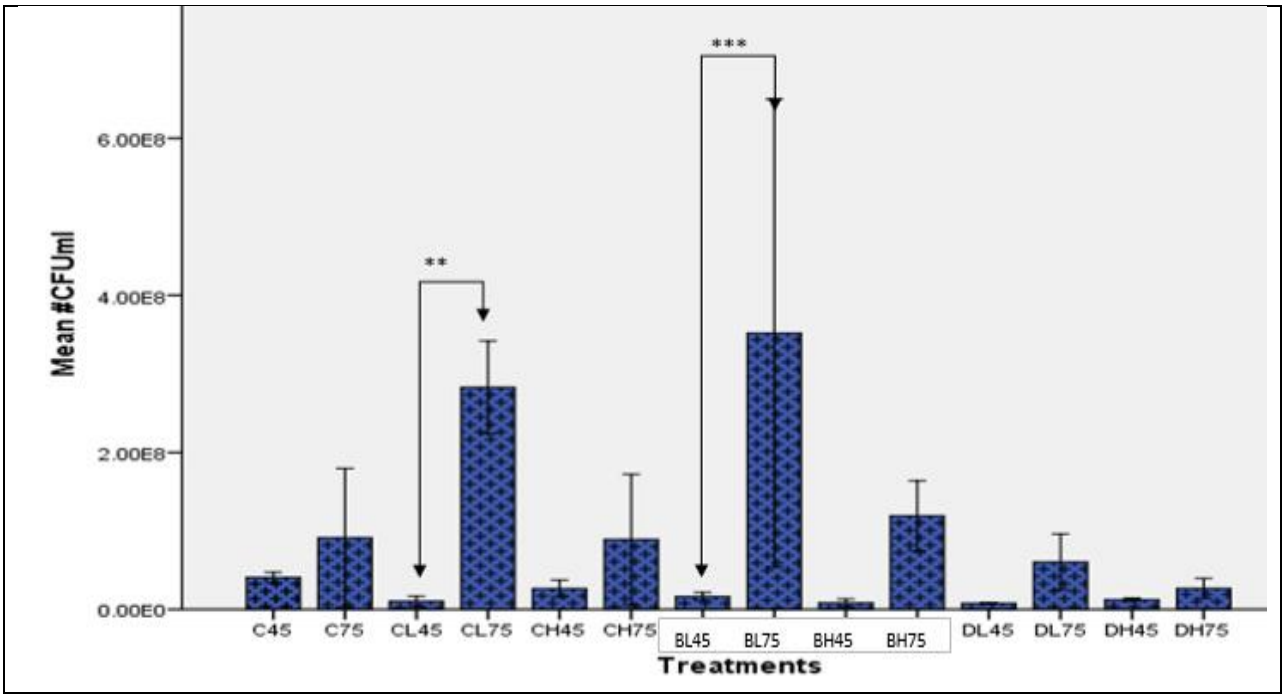

Figure 11:Difference in average CFU counts of cultured rhizosphere bacteria on both media (RIM media and dRSM) between both time point of post pesticide treatment C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) at two-time point for each treatment are represented by *. Error bars represent standard

The cultured Rhizospheric bacterial population increased at 75 days post treatment compared with 45 days post treatment. Rhizospheric bacterial communities have competent systems for uptake and catabolism of organic compounds present in root exudates (Tilak et al., 2005). Plants are constantly secreting root exudates and they are considered the key factor for the supplementation of specific microbial populations in the rhizosphere (Berg and Smalla, 2009) which could be the reason for the observed increase in bacterial growth.

Abundance of total culturable bacteria on both media showed a significant reduction at initial sampling points for all pesticide treatment except for Confidor high dose and, Bogiron low dose on RIM media. However, at the second time point on RIM, Confidor high dose and Bogiron (both doses) enhance bacterial growth compared with the control. The same pattern is observed with the

cultured bacteria on dRSM for all treatments except for Durspan high dose, where the cultured bacteria were sensitive to the high dose at both time point compared with the control. Such a recovery from inhibition could be attributed to the ability of microorganisms to adapt to various environmental stress (Walvekar et al., 2017). However, the relationship between the chemical structure of pesticide and its effect on the different groups of soil microorganisms is not easy to be predicted. Some pesticides have depressive effects or no effects on microorganisms when applied at normal rates but other pesticides can stimulate the growth of microorganisms, (Lo, 2010, Hussain et al., 2009). Confidor and Bogiron, when applied at recommended doses, show to stimulate the growth of cultured rhizospheric bacteria, while the high dose shows no significant differences when compared to the control. The inhibitory effects of the pesticide Chlorpyrifos was studied by (Wang et al., 2010) through the measurement of metabolic parameters and the microbial urease enzyme,. Their study shows that when soil microorganisms were exposed to chlorpyrifos at different doses, their metabolic activities were suppressed to different extents., This illustrates that individual microorganisms reacted to stress from environment change by shifting resources from other biological activities (such as reproduction or growth) toward survival. Another study by (Singh et al., 2015a) found that chlorpyrifos at the higher dose inhibited *Pseudomonas spp*., a study by(Ahmed and Ahmad, 2006) showed that chlorpyrifos caused significant reduction in the number of soil bacteria.

7.5 Nitrogen fixing bacteria

Nitrogen fixing bacteria are free-living bacteria, which grow well on a nitrogen free medium. Rhizospheric bacteria from soil were inoculated on nitrogen free medium (Burks medium) for the detection of nitrogen fixing bacteria the extraction dilution was 10^4. Figure 12 represent the growth of nitrogen fixing bacteria at both time points under different treatments. Table 8 shows the CFU count for the cultured bacteria. There was a significant difference in the CFU after the application of pesticide at both doses and at both time points. One-way ANOVA: post Hoc multiple comparisons Tukey test (Table A6 see Annex) result was carried out to identify significant differences between treatments compared with the control, significant differences between both doses, and the significant differences between both time points.

Table 8: number of N_2 fixing cultured rhizosphere bacteria colony forming units at both time point of post pesticide treatment the extraction dilution was 10^4 for both time. (3 replicate for each treatment and was plated in duplicate).

N_2 fixing bacteria #CFU 45 day			**N_2 fixing bacteria #CFU 75 day**		
Treatments	Mean	Std. Deviation	Treatments	Mean	Std. Deviation
C45	2.47E+07	7.50E+06	C75	3.85E+07	9.19E+06
CL45	1.14E+07	3.82E+06	CL75	6.00E+07	1.41E+07
CH45	4.70E+06	1.84E+06	CH75	2.76E+07	2.05E+06
BL45	6.65E+06	4.95E+05	BL75	4.45E+07	6.36E+06
BH45	6.85E+06	2.19E+06	BH75	3.08E+07	7.71E+06
DL45	5.50E+05	6.36E+05	DL75	3.00E+05	1.41E+05
DH45	2.35E+06	7.07E+04	DH75	3.50E+05	2.12E+05
p-value		0.003		0.001	

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

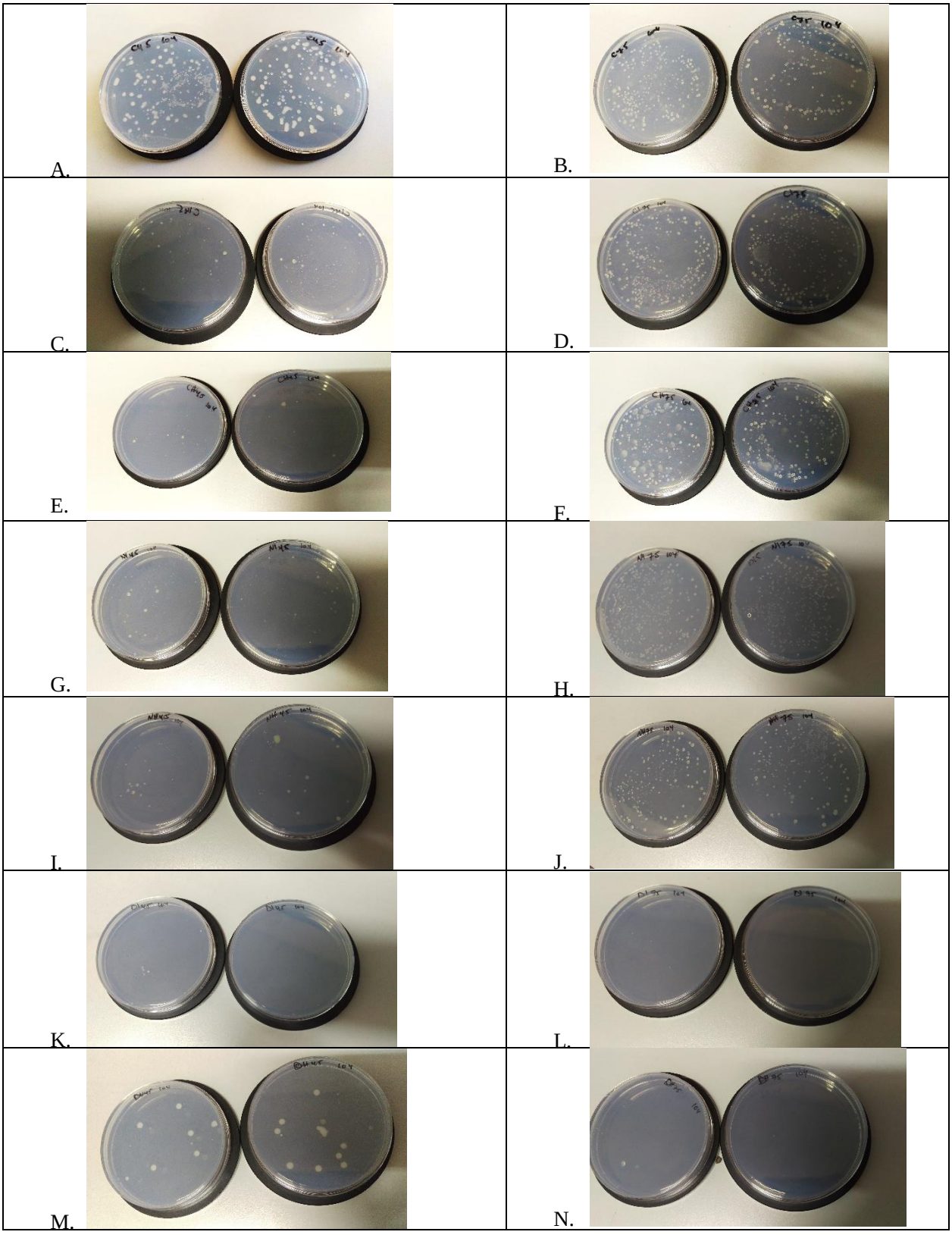

Figure 12:cultured N2 fixing rhizospheric bacteria on both time point of post pesticide treatment .A) C45, B) C75, C) CL45, D) CL75, E) CH45, F) CH75, G) BL45, H) BL75, I) BH45, J) BH75, K) DL45, L) DL7, M) DH45, N) DH75.C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

A significant reduction in nitrogen fixing bacterial communities was observed under all pesticide treatments (Confidor low dose (p=0.051), Confidor high dose (p=0.006), Bogiron low dose (p=0.011), Bogiron high dose (p=0.012), Durspan low dose (p=0.002), Durspan high dose (p=0.003) at first time point when compared to control soil, with maximum reduction by both dosages of Durspan (Figure 13. I). There was no significant difference between both doses for each type of pesticide. At the second time point there was no significant difference between both doses of Confidor and Bogiron compared to the control, but a significant reduction by both dosages of Durspan (p=0.014) (Figure 13. II), data also show that there was a significant a reduction in nitrogen fixing bacterial communities at high dose of Confidor compared with the low dose (p=0.032).

Figure 14 represents the comparison between the number of CFU of N_2 fixing bacteria at both time points for each treatments. There was a significant increase in CFUs at the second time point for Confidor high dose (p=0.053), Confidor low dose (p=0.053), Bogiron low dose (p=0.001), and Bogiron high dose (p=0.039) compared with the first time point. There were no differences in CFU number for the other treatment at both time points.

Atmospheric nitrogen becomes available to plants when nitrogen fixing bacteria fix atmospheric nitrogen and convert it into ammonia (NH_3) in the presence of the enzyme nitrogenase. The results show that N_2 fixing bacteria were highly sensitive to the application of pesticide compared to the control, where there was a significant reduction at the first time point compared to the control. The application of Durspan was lethal for nitrogen fixers on both time point and both doses. For the other two pesticides at the second time point there was no difference compared with the control. A similar study done by Walvekar (Walvekar et al., 2017) shows that nitrogen fixers were the most deleteriously affected group upon pesticide application (Walvekar et al., 2017). The action of

pesticides on nitrogen fixation was analyzed also by a study using cultivation-independent analysis (Singh et al., 2015a). In that study the active bacterial community involved in nitrogen fixation was disturbed toward the later stage of the legume growth as demonstrated by the reduced number of *nifH* transcripts (which includes the conversion of atmospheric nitrogen into ammonia) with the application of chlorpyrifos at both doses (Singh et al., 2015a).Another study done by (Fox et al., 2007) showed that a one-time treatment with some natural and synthetic environmental chemicals is sufficient to significantly inhibit nodule formation and nitrogenase activity in all chemical treatment groups compared with the control . They suggested that this may be due to the fact that natural phytochemical and synthetic agrichemicals hinder the symbiotic signaling between host plants and neighboring bacterial community as shown in the rhizosphere of alfalfa plant.

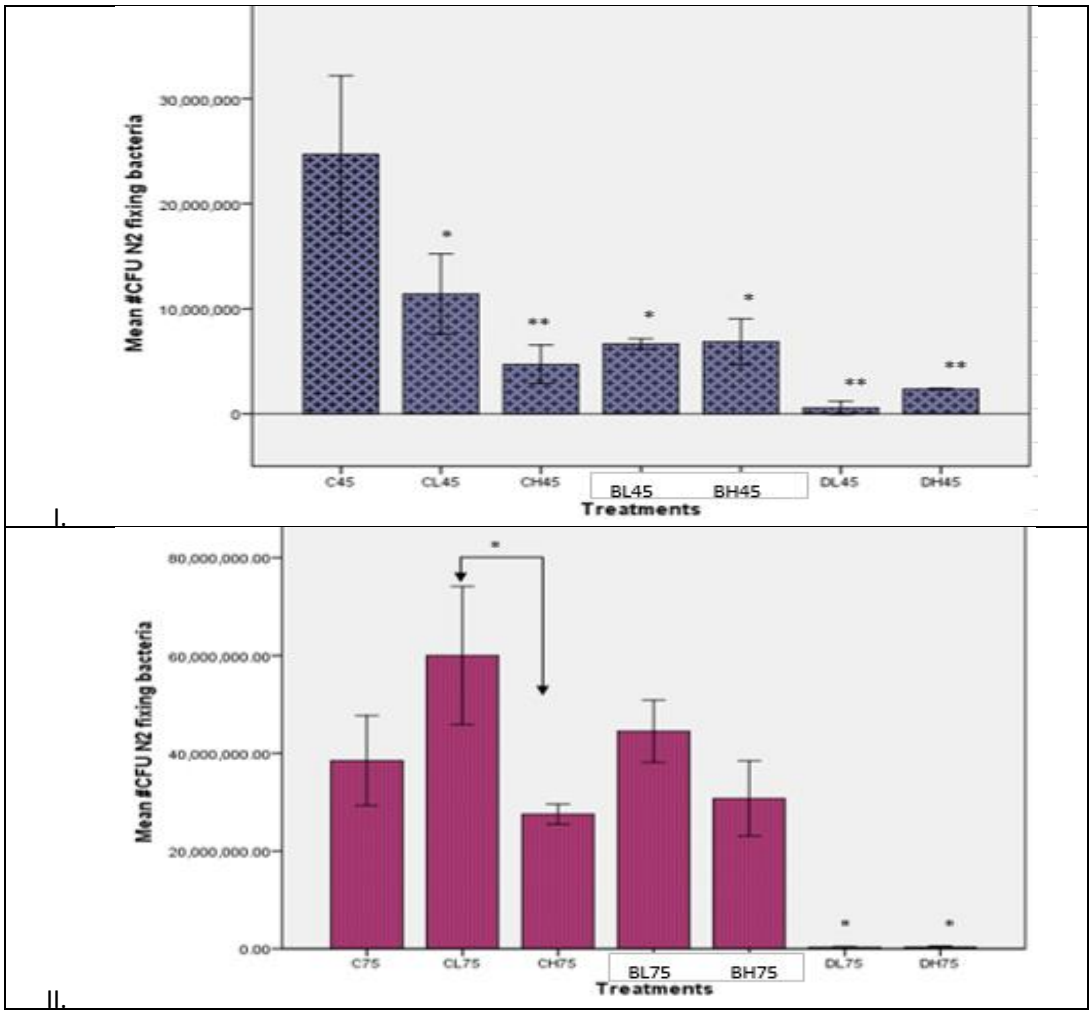

Figure 14: Difference in CFU of N2 fixing cultured rhizosphere bacteria after I) 45 day, II) 47 day of post pesticide treatment. C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) between the control and the treatments are representing by *. Error bars represent standard deviations

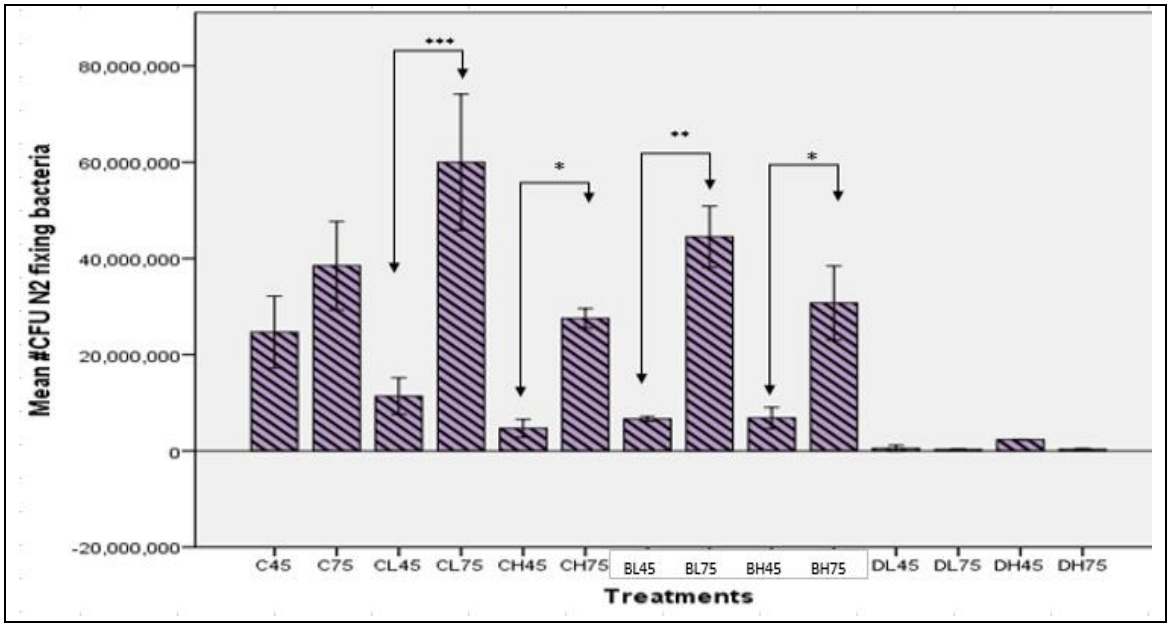

Figure 13: Difference in CFU of cultured N2 fixing rhizospheric bacteria between both time point of post pesticide treatment. C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose. Significantly different values ($P < 0.05$) at two-time point for each treatment are represented by *. Error bars represent standard deviations.

7.6 Isolation and identification of cultured rhizosphere microbial community

Colonies from both media (RIM and dRSM) shown in Fig 5 and 6 were isolated to determine their presence or absence in all treatments. On RIM media at both time points there were 11 different colony isolates (Table A7 see Annex). Three isolates: R1, R2 and R4 were present in all treatments and in the control at both time points. Isolates R6 and R13 were not present in the control at the first time point, R13 was also not present at the second time point together with R7 and R14. (Table A7 see Annex) shows all isolates in relationship to their presence or absence under different treatment and at different time points.

On dRSM medium at both time points there were 28 different colony isolates (Table A8 see Annex). 6 isolates (D1, D6, D7, D8, D12, and D21) were present in all treatments and in the control for both time points. 8 isolates (D9, D11, D13, D14, D16, D24, D25, and D28) were not found in the control for both time points. 6 isolates were absent at the first time point (D2, D3, D5, D10, D20, and D22) but were present at the second time point, and 4 isolates (D4, D15, and D26) were present at the first time point but they were absent at the second time point (Table A8 see Annex) shows the presence or absence of the isolates on dRSM medium.

Each one of those isolates from both media was re-streaked on Trypticase soy agar media for identification purposes. Preliminary characterization was performed using morphological characteristics (Table A9 see Annex). Morphological characterization was based on classical macroscopic observations of pure colony color, margin, texture, elevation, and surface. Most colonies were able to grow within 2-3 days of incubation at 28 ºC. There was a variation in the colony morphology of the isolates. The gram staining of the cell and their shape was determined under the microscope (Table A9 and Table A10 see Annex).

The ability of the isolates to excrete extracellular enzymes was tested through hydrolysis of starch, and gelatine. The presence of intracellular enzymes was determined through tests such as catalase reaction; urease, methyl red-Voges Proskauer, hydrogen sulphide production, nitrate reduction, methyl red, phosphate solubilization, nitrogen fixation, citrate utilization, oxidase, and motility. The isolates differed greatly on their activity of secreted and intracellular enzymes (Table A11 see Annex). The sensitivity of the isolates to Amoxicillin and Vancomycin was also tested to identify presence or absence of peptidoglycan.

Gram negative bacteria were plated on *Pseudomonas* selective agar to test whether they could be assigned to this genus or not. 12 isolates (R1, R11, R13, R14, D5, D17, D18, D19, D20, D21, D25and, D27) grew on this medium and could be assigned to *Pseudomonas spp*,. 4 isolates were identified to belong to *Actinobacteria spp.* according to their morphology (R5, R7, D11, and D24). 3 isolates were confirmed to belong to *Bacillus spp.* by inoculation on *Bacillus cereus* selective agar: D13, and D15 appeared as a pink colony after inoculation on the medium which confirmed them as *Bacilluscereus*; D28 produces a yellow color in the medium which identifies it as *Bacillus subtilis*. D7 and D9 were assigned to be *Staphylococcus sp* according to their morphology and cell staining.

Most of the gram negative bacteria belonged to *Pseudomonas sp*. For the gram positive rod shape isolates, 7 of them were identified to be *Actinobacteria spp.* and *Bacillus spp*, the coccus shape gram positive cells were identified to be *Staphylococcus spp.* Another study determined that the bacterial strains that are predominant in the rhizosphere contain gram-negative, rod shaped, non-sporulating bacteria that belong to the group *Proteobacteria*, with *Pseudomonas*as the most common (Antoun and Prévost, 2005, Antoun et al., 1998). This may be because of the effectiveness of the gram negative bacteria's consumption of root exudates, combined with stimulation by rhizo-

deposition; the growth of gram positive bacteria is generally inhibited (Steer and Harris, 2000). The gram-positive, rods or cocci and spore forming strains like *Bacillus* are relatively less numerous and varied, strains of *Bacillus* are considered the main inhabitants gram-positive bacteria of the rhizosphere (Prashar et al., 2014, Antoun et al., 1998)

Various rhizospheric bacteria are marketed as biological control agents including the genera *Bacillus, Streptomyces* , and *Pseudomonas* (Saharan and Nehra, 2011). They protect from plant disease at least by different mechanisms, such as induction of systemic resistance, and production of antibiotics or siderophores (Saharan and Nehra, 2011). Some *Bacillus* species have been classified as plant growth promoting Rhizobacteria (Probanza et al., 2002), *Bacillus* is the most abundant gram positive genus in the rhizosphere; these bacteria release a number of metabolites which affect the environment by increasing nutrient availability to the plants (Antoun and Prévost, 2005, Saharan and Nehra, 2011)

Pseudomonas spp. are considered universal bacteria in agricultural soils and have many traits that make them well suited as PGPR (Saharan and Nehra, 2011). Fluorescent *Pseudomonas spp.*are the most effective strains of *Pseudomonas*, they help in the conservation of soil health andare functionally and metabolically most diverse (Lugtenberg and Dekkers, 1999, Saharan and Nehra, 2011).

Actinobacteria, are universal soil bacteria known for the production of a various range of bioactive secondary metabolites, they are also identified as an important group of microbes that inhabit the plant rhizosphere , they were described as biocontrol agents of several economically important plant pathogens (Arunachalam Palaniyandi, (2013).)

The nitrogen fixation test was done to test the ability of the isolates to fix nitrogen. Most of the isolates were able to fix the atmospheric nitrogen by enzyme nitrogenase, which reduces nitrogen to ammonia; nitrogen is a key component in soil fertility. The ability of the isolates to reduce nitrate indicates their ability to produce enzyme nitrate reductase which reduces nitrates that the cell uses as a final hydrogen acceptor during anaerobic respiration to nitrites or free nitrogen gas and water (Wafula et al., 2015, Saharan and Nehra, 2011). This is an important factor to help maintain the nitrogen cycle (Wafula et al., 2015).

Phosphate solubilizing bacteria (PSB) are the group of common PGPR in rhizosphere (Chen et al., 2006). This group of bacteria are able to secrete organic acids and phosphatases to solubilize insoluble phosphate to soluble forms (Chaiharn et al., 2008).Phosphate solubilization is equally important as nitrogen fixation, because Phosphorus (P) is considered to be one of the major essential macronutrients for biological development and growth (Chen et al., 2006). Most of the isolate resulted positive for this test.

8. Conclusion

In conclusion, the present study aimed to study the effects of 3 pesticides ((Confidor (Imidacloprid), Bogiron (Difenoconazole) and, Durspan (Chlorpyrifos)) used by Palestinian farmers and assess their effect on cultured rhizospheric bacterial communities and on N_2 fixing bacteria on *Vicia faba* plants at two time points (45 and 75 days post-treatment) and two dosages (low dose and high dose).

This study was the first to study the non-target effect of the 3 pesticides ((Confidor (Imidacloprid), Bogiron (Difenoconazole) and, Durspan (Chlorpyrifos)) on cultured rhizospheric bacterial communities of *Vicia faba* plants.

- Plant parameters were not affected significantly by Confidor and Bogiron compared with the control, whereas Durspan affected the plant root and burned the leaves.
- The three pesticides studied differed widely in their degradation behavior, where Bogiron show a slower degradation than Confidor and Durspan.
- All treatments show a significant decrease on the average number of CFUs at the first time point when compared with the control; this decrease in CFUs is not found for all treatments at the second time point. The CFUs count for each treatment between both time points shows that the application of low dose of Confidor and Bogiron enhanced the growth of cultured rhizospheric bacteria with time.
- A significant reduction in the nitrogen fixing bacterial community was observed under all pesticide treatments at the first time point compared with the control with maximum reduction by both dosages of Durspan. Both dosages of Durspan show significant reduction also at the second time point. A significant increase in N_2 fixing bacteria CFUs is recorded

for both doses of Confidor and Bogiron at the second time point when compared with the first time point.

- Confidor and Bogiron show to enhance the growth of cultured rhizospheric bacteria with time, but Durspan shows inhibition of the growth of cultured rhizospheric bacteria with time.
- A total of 39 different colonies were counted on RIM, and dRSM media. These colonies were isolated to be identified according to their morphological characteristic and biochemical test.
- 77% of the isolates from 39 isolate show their ability to fix nitrogen.

The results of this study confirm that pesticides show non-target effects on active microbial populations that attend important ecosystem functions by either enhancing or inhibiting their growth. There is a need to further study the effect of pesticides on non-target organisms and to optimize the use of pesticides in agriculture before accepting them. This study also recommends prohibiting the use of Durspan as insecticide on *Vicia faba.* Also there is a need to educate farmers on the use of pesticides and to reemphasize the significance of recommended dosages

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10. Annex

Table A1: one-way ANOVA: post Hoc multiple comparisons Tukey test result comparing plants parameters after both time point of post pesticide treatment

Multiple Comparisons			
Tukey HSD			
Dependent Variable			Sig.
Root length	C75	BH75	0.983
		BL75	0.699
		DL75	0.585
		DH75	**0.031**
		CL75	0.864
		CH75	0.864
	BH75	C75	0.983
		BL75	0.983
		DL75	0.949
		DH75	0.125
		CL75	0.999
		CH75	0.999
	BL75	C75	0.699
		BH75	0.983
		DL75	1
		DH75	0.403
		CL75	1
		CH75	1
	DL75	C75	0.585
		BH75	0.949
		BL75	1
		DH75	0.509
		CL75	0.998
		CH75	0.998
	DH75	C75	0.031
		BH75	0.125
		BL75	0.403
		DL75	0.509
		CL75	0.257

		CH75	0.257
	CL75	C75	0.864
		BH75	0.999
		BL75	1
		DH75	0.998
		DL75	0.257
		CH75	1
	CH75	C75	0.864
		BH75	0.999
		BL75	1
		DH75	0.998
		DL75	0.257
		CL75	1
Dry matter	C75	BH75	0.999
		BL75	0.999
		DL75	0.625
		DH75	0.302
		CL75	1
		CH75	0.916
	BH75	C75	0.999
		BL75	0.963
		DL75	0.381
		DH75	0.155
		CL75	1
		CH75	0.993
	BL75	C75	0.999
		BH75	0.963
		DL75	0.873
		DH75	0.547
		CL75	0.97
		CH75	0.694
	DL75	C75	0.625
		BH75	0.381
		BL75	0.873
		DH75	0.995
		CL75	0.401
		CH75	0.142
	DH75	C75	0.302

		BH75	0.155
		BL75	0.547
		DL75	0.995
		CL75	0.165
		CH75	0.049
	CL75	C75	1
		BH75	1
		BL75	0.97
		DH75	0.401
		DL75	0.165
		CH75	0.99
	CH75	C75	0.916
		BH75	0.993
		BL75	0.694
		DH75	0.142
		DL75	**0.049**
		CL75	0.99

***. The mean difference is significant at the 0.05 level.**

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Table A2: one-way ANOVA: post Hoc multiple comparisons Tukey test result comparing Pesticide residual concentration in soil after both time point of post pesticide treatment

Multiple Comparisons		
Dependent Variable: Pesticide concentration Tukey HSD		
pesticide residual		Sig.
CL45	CL75	1.000
	CH45	.896
CH45	CH75	1.000
	CL45	.896
BL45	BL75	1.000
	BH45	.780
BH45	BH75	.195
	BL45	.780
DL45	DH75	1.000
	DH45	**.002**
DH45	DH75	**.000**
	DL45	**.002**
CL75	CL45	1.000
	CH75	.997
CH75	CH45	1.000
	CL75	.997
BL75	BL45	1.000
	BH75	**.003**
BH75	BH45	.195
	BL75	**.003**
DL75	DL45	.900
	DH75	.999
DH75	DH45	**.000**
	DL75	.999

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Table A3: One-Sample T- Test for the rhizospheric soil pH between the two-time points

One-Sample Test					
Sample	t	df	Sig. (2-tailed)	95% Confidence Interval of the Difference	
				Lower	Upper
Soil pH after 45 day	408.875	18	**0.000**	7.8175	7.8983
Soil pH after 75 day	179.092	18	**0.000**	7.0739	7.2419

Table A4: one-way ANOVA: post Hoc multiple comparisons Tukey test result comparing rhizosphere bacteria colony forming units on RIM media and dRSM media at both time point of post pesticide treatment

Multiple Comparisons							
Tukey HSD							
Dependent Variable			Sig.	**Dependent Variable**			Sig.
#CFU\ml RIM media 45 day	C45	CL45	**.012**	**#CFU\ml dRSM media 45 day**	C45	CL45	**.000**
		CH45	1.000			CH45	**.001**
		BL45	.126			BL45	**.000**
		BH45	**.016**			BH45	**.000**
		DL45	**.008**			DL45	**.000**
		DH45	**.019**			DH45	**.000**
	CL45	C45	**.012**		CL45	C45	**.000**
		CH45	**.015**			CH45	.638
	CH45	C45	1.000		CH45	C45	**.001**
		CL45	**.015**			CL45	.638
	BL45	C45	.126		BL45	C45	**.000**
		BH45	.577			BH45	.535
	BH45	C45	**.016**		BH45	C45	**.000**
		BL45	.577			BL45	.535
	DL45	C45	**.008**		DL45	C45	**.000**
		DH45	.972			DH45	.830
	DH45	C45	**.019**		DH45	C45	**.000**
		DL45	.972			DL45	.830
#CFU\ml RIM media 75 day	C75	CL75	**.000**	**#CFU\ml dRSM media 75 day**	C75	CL75	.090
		CH75	1.000			CH75	1.000
		DL75	.995			DL75	.072
		DH75	1.000			DH75	**.005**
		BL75	**.000**			BL75	.088
		BH75	**.024**			BH75	.098
	CL75	C75	**.000**		CL75	C75	.090
		CH75	**.000**			CH75	.059
	CH75	C75	1.000		CH75	C75	1.000
		CL75	**.000**			CL75	.059
	DL75	C75	.995		DL75	C75	.072
		DH75	.997			DH75	.285
	DH75	C75	1.000		DH75	C75	**.005**
		DL75	.997			DL75	.285
	BL75	C75	**.000**		BL75	C75	.088

		BH75	**.000**			BH75	1.000
	BH75	C75	**.024**		BH75	C75	.098
		BL75	**.000**			BL75	1.000
#CFU\ml RIM both time points	C45	C75	0.995	**#CFU\ml dRSM** both time points	C45	C75	**0**
	CL45	CL75	**0**		CL45	CL75	**0**
	CH45	CH75	0.999		CH45	CH75	**0**
	BL45	BL75	**0**		BL45	BL75	0.**005**
	BH45	BH75	**0.001**		BH45	BH75	**0.002**
	DL45	DL75	0.992		DL45	DL75	**0.004**
	DH45	DH75	1		DH45	DH75	0.917

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Table A5: one-way ANOVA: post Hoc multiple comparisons Tukey test result comparing avg rhizosphere bacteria colony forming units from both RIM media and dRSM media at both time point of post pesticide treatment

Multiple Comparisons		
Dependent Variable **Tukey HSD**		
Avg #CFU\ml from both media 45 day		Sig.
C45	CH45	**.044**
	CL45	**.000**
	BL45	**.000**
	BH45	**.000**
	DL45	**.000**
	DH45	**.000**
CH45	C45	**.044**
	CL45	**.020**
CL45	C45	**.000**
	CH45	**.020**
BL45	C45	**.000**
	BH45	.535
BH45	C45	**.000**
	BL45	.535
DL45	C45	**.000**
	DH45	.942
DH45	C45	**.000**
	DL45	.942
Avg #CFU\ml from both media 75 day		Sig.
C75	CH75	1.000
	CL75	.359
	DL75	1.000
	DH75	.989
	BL75	.095
	BH75	1.000
CH75	C75	1.000
	CL75	.347
CL75	C75	.359
	CH75	.347
DL75	C75	1.000
	DH75	1.000

DH75	C75	.989
	DL75	1.000
BL75	C75	.095
	BH75	.171
BH75	C75	1.000
	BL75	.171
Avg #CFU\ml from both media both time points		Sig.
C45	C75	1.000
CH45	CH75	.999
CL45	CL75	**.006**
BL45	BL75	**.000**
BH45	BH75	.880
DL45	DL75	1.000
DH45	DH75	1.000

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

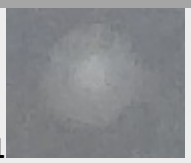
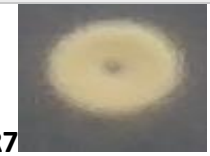
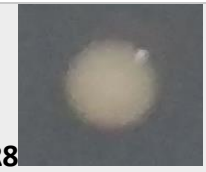
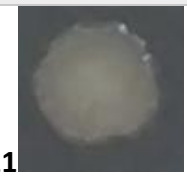
Table A6: one-way ANOVA: post Hoc multiple comparisons Tukey test result comparing rhizosphere bacteria colony forming units for N2 fixing bacteria at both time point of post pesticide treatment.

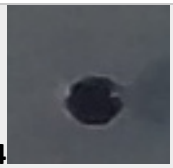
Multiple Comparisons		
Tukey HSD		
Dependent Variable		
N2 fixing bacteria #CFU/ml 45 day		Sig.
C45	CH45	**.006**
	CL45	**.051**
	BH45	**.012**
	BL45	**.011**
	DH45	**.003**
	DL45	**.002**
CH45	C45	**.006**
	CL45	.491
CL45	C45	**.051**
	CH45	.491
BH45	C45	**.012**
	BL45	1.000
BL45	C45	**.011**
	BH45	1.000
DH45	C45	**.003**
	DL45	.997
DL45	C45	**.002**
	DH45	.997
C75	CL75	.181
	CH75	.754
	BL75	.977
	BH75	.928
	DL75	**.014**
	DH75	**.014**
N2 fixing bacteria #CFU/ml 75 day		**Sig.**
CL75	C75	.181
	CH75	**.032**
CH75	C75	.754
	CL75	**.032**
BL75	C75	.977
	BH75	.561
BH75	C75	.928

	BL75	.561
DL75	C75	**.014**
	DH75	1.000
DH75	C75	**.014**
	DL75	1.000
N2 fixing bacteria #CFU/ml both time points		Sig.
C45	C75	.529
CH45	CH75	**.053**
CL45	CL75	.000
BH45	BH75	**.039**
BL45	BL75	**.001**
DH45	DH75	1.000
DL45	DL75	1.000

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

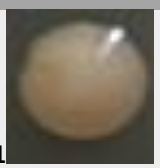
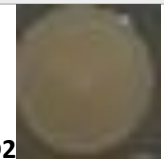
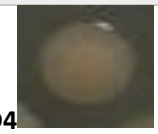
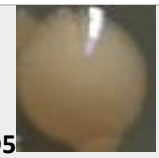
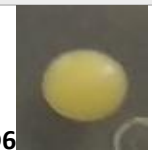
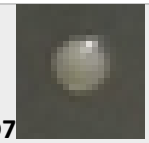
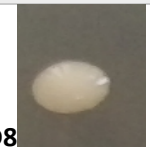
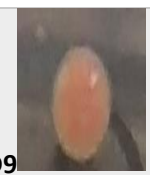
Table A7: Cultured rhizosphere bacteria on RIM medium presence or absence after pesticides treatment at both time points.

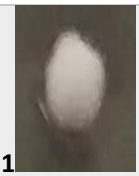
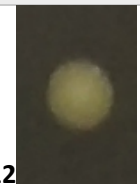
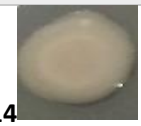
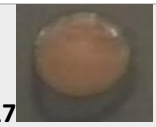
Colony Isolate	C45	CL45	CH45	BL45	BH45	DL45	DH45	C75	CL75	CH75	BL75	BH75	DL75	DH75
 R1	X	X	X	X	X	X	X	X	X	X	X	X	X	X
 R2	X	X	X	X	X	X	X	X	X	X	X	X	X	X
 R4	X	X	X	X	X	X	X	X	X	X	X	X	X	X
 R5	X	X	X	X	X	X	X	X			X			X
 R6				X				X						
 R7	X	X	X	X						X				
 R8	X	X		X	X	X		X	X	X	X	X	X	X
 R11	X	X	X	X	X	X	X	X	X	X	X	X		

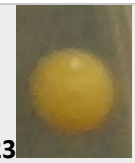
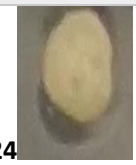
R12 	X	X	X	X	X		X	X	X	X				
R13 		X	X		X		X				X			
R14 	X		X											
Total	9	9	9	9	8	6	7	8	6	7	7	5	4	5

C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Table A8: Cultured rhizosphere bacteria on dRSM medium presence or absence in each pesticide treatment at both time point

Colony type	C45	CL45	CH45	BL45	BH45	DL45	DH45	C75	CL75	CH75	BL75	BH75	DL75	DH75
D1 	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D2 		X		X	X	X	X	X	X	X	X	X	X	
D3 		X	X	X	X	X	X	X	X		X	X		X
D4 	X	X	X	X		X	X				X	X		
D5 			X		X	X	X	X	X	X	X	X		
D6 	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D7 	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D8 	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D9 		X		X		X	X					X	X	

D10 		X			X			X			X	X		
D11 		X		X										
D12 	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D13 				X										
D14 		X	X	X		X					X		X	
D15 	X					X					X			
D16 				X										
D17 	X	X	X	X	X	X	X	X	X	X	X			
D18 	X							X					X	
D19 	X	X				X		X	X	X	X	X	X	

D20 						X		X				X		
D21 	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D22 								X						
D23 								X				X		
D24 				X										
D25 						X							X	
D26 	X													
D28 							X							
Total = 27	12	15	10	16	11	17	13	16	11	10	15	15	12	7

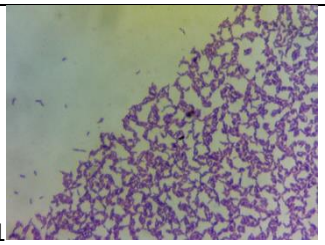
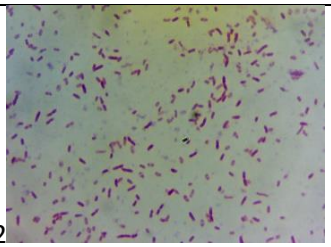
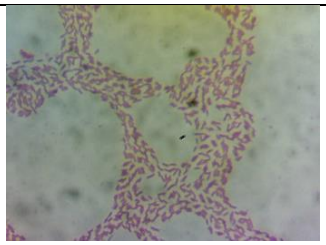
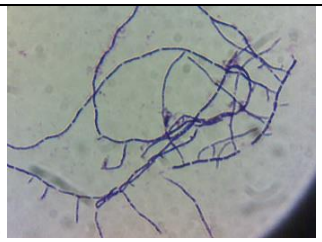
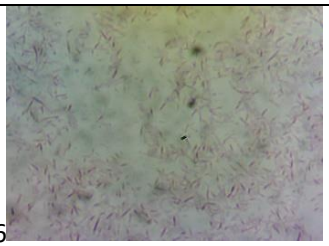
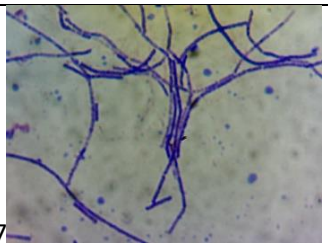
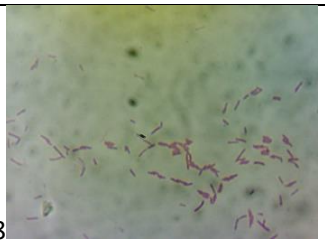
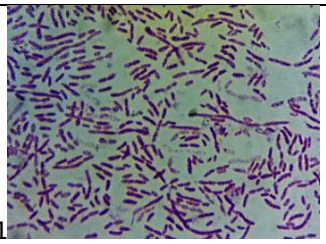
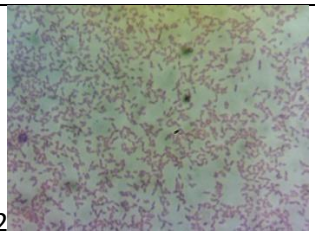
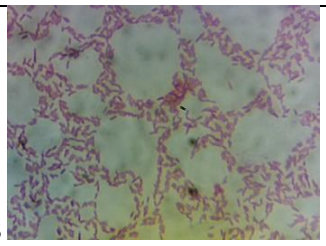
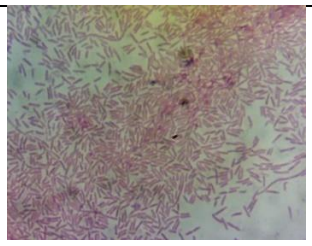
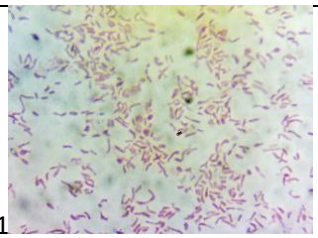
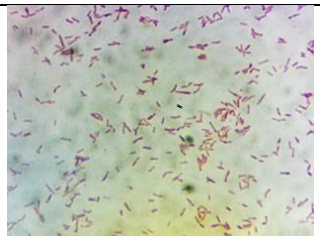
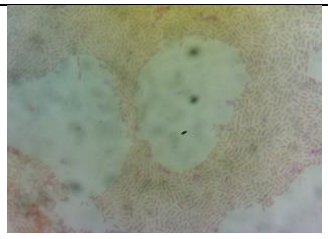
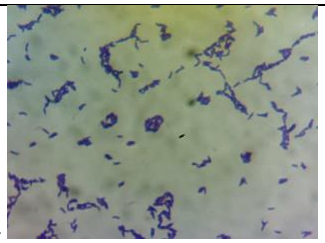
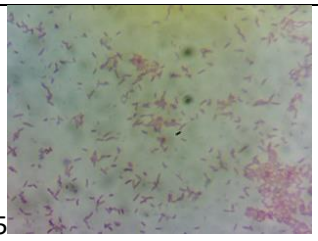
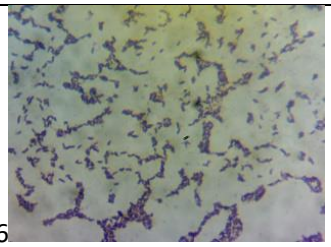
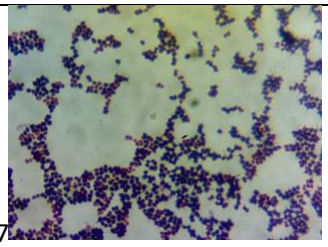
C= Control, CL= Confidor low dose, CH= Confidor high dose, BL= Bogiron low dose, BH= Bogiron high dose, DL= Durspan low dose, DH= Durspan high dose.

Table A9: Morphological characteristics of isolates of cultured rhizosphere bacteria from both media (RIM and DRSM) on Trypticase Soy Agar

SAMPLE	GRAM STAIN	SHAPE	COLONY COLOR	COLONY MARGIN	CONSISTENCY or TEXTURE	ELEVATION OF COLONY	SURFACE OF COLONY
R1	G-NEGATIVE	ROD	Cream	Entire	Butyrous	Flat	Glistening
R2	G-POSITIVE	ROD	Cream	Entire	Mucoid	Raised	Viscous
R4	G-NEGATIVE	Rod	Cream	Entire	Butyrous	Flat	Glistening
R5	G-POSITIVE	Filament	Cream	Entire	dry	Growth into medium+ raised	Fried egg
R6	G-POSITIVE	Rod	White/yellow	Entire	Mucoid	Flat	Glistening
R7	G-POSITIVE	Filament	Pink	Entire	Dry	Growth into medium+ raised	Rough / wrinkled
R8	G-POSITIVE	Rod	Yellow	Entire	Mucoid	Raised	Glistening
R11	G-NEGATIVE	Rod	White	Entire	Mucoid	Raised	Viscous
R12	G-NEGATIVE	Rod	Yellow	Entire	Mucoid	Raised	Viscous
R13	G-NEGATIVE	Rod	Cream	Entire	Butyrous	Flat	Glistening
R14	G-NEGATIVE	Rod	Cream	Entire	Butyrous	Flat	Glistening
D1	G-NEGATIVE	Rod	Cream	Entire	Mucoid	Flat	Viscous
D2	G-POSITIVE	Rod	Cream	Entire	Mucoid	Raised	Viscous
D3	G-NEGATIVE	Rod	Orange (orangered)	Entire	Mucoid	Flat	Viscous
D4	G-POSITIVE	Rod /comma	White	Entire	Butyrous	Flat	Glistening
D5	G-NEGATIVE	Rod	Cream	Entire	Mucoid	Raised	Viscous
D6	G-POSITIVE	Rod small	Yellow	Entire	Mucoid	Flat	Viscous
D7	G-POSITIVE	Cocci	Mustard	Entire	Butyrous	Flat	Glistening
D8	G-POSITIVE	Rod	White	Entire	Dry	Flat	Dull
D9	G-POSITIVE	Cocci	Pink Orange	Entire	Mucoid	Flat	Viscous
D10	G-NEGATIVE	Rod	Cream	Entire	Butyrous	Flat	(wrinkled)+ glistening
D11	G-POSITIVE	Filament	Cream	Irregular	Dry	Growth into medium+ raised	Rough
D12	G-POSITIVE	Rod small	Yellow	Entire	Mucoid	Flat	Viscous

D13	G-POSITIVE	Rod large/endospore	Cream	Irregular	Dry	Flat	rugose (wrinkled)
D14	G-POSITIVE	Rod large	Cream	Entire	Butyrous	Flat	Glistening
D15	G-POSITIVE	Rod /endospore	Cream	Irregular	Dry	Flat	rugose (wrinkled)
D16	G-NEGATIVE	Rod	Yellow/ orange	Entire	Butyrous	Flat	Glistening
D17	G-NEGATIVE	Rod	Cream	Entire	Butyrous	Flat	Glistening
D18	G-NEGATIVE	Rod	Light pink	Entire	Butyrous	Flat	Dull (opposite of glistening)
D19	G-NEGATIVE	Rod	Orange	Entire	Butyrous	Flat	Glistening
D20	G-NEGATIVE	Rod	Cream	Entire	Butyrous	Flat	Glistening
D21	G-NEGATIVE	Rod	Cream	Entire	Mucoid	Flat	Viscous
D22	G-NEGATIVE	Rod	Cream	Entire	Mucoid	Flat	Viscous
D23	G-POSITIVE	Rod small /endospore	Yellow	Entire	Mucoid	Flat	Glistening
D24	G-POSITIVE	Rod	Cream	Irregular	Dry	Growth into medium +raised	rough, (stuck to the agar media)
D25	G-NEGATIVE	Rod small	Cream	Irregular	Dry	Raised	rugose (wrinkled)
D26	G-POSITIVE	Rod	Cream	Filamentous	Mucoid	Umbonate	rugose (wrinkled)+dull
D27	G-NEGATIVE	Rod	Cream	Entire	Mucoid	Flat	Viscous
D28	G-POSITIVE	Rod/ endospore	cream	Entire	Butyrous	Flat	Glistening

Table A10: Gram staining of the cell and their shape under microscope of cultured rhizosphere bacteria from both media (RIM and DRSM) after streaking on Trypticase Soy Agar.

R1 	R2 	R4 
R5 	R6 	R7 
R8 	R11 	R12 
R13 	R14 	D1 
D2 	D3 	D4 
D5 	D6 	D7 

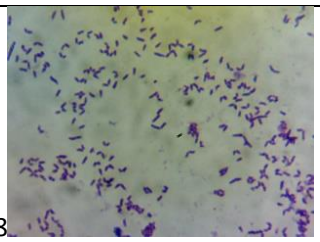
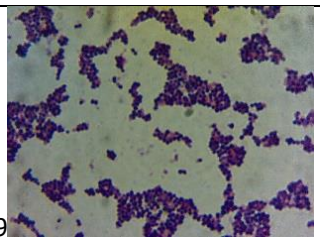
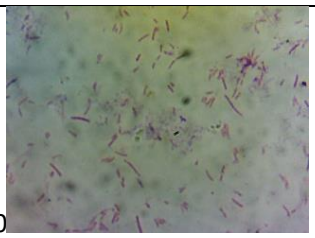
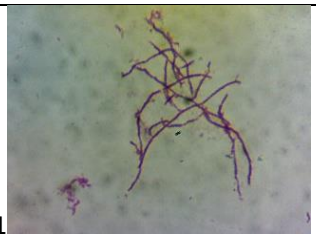
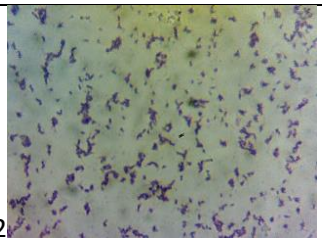
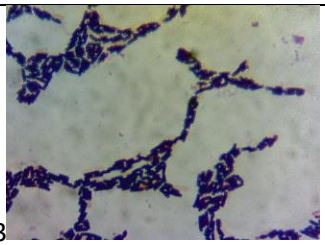
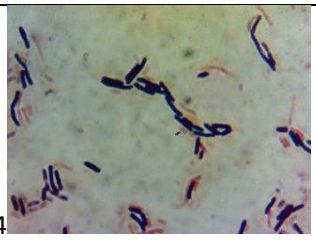
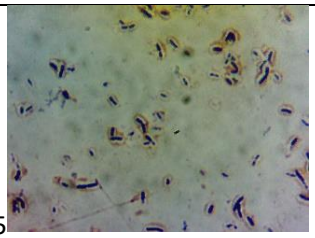
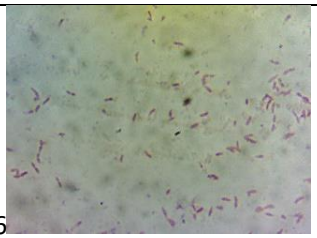
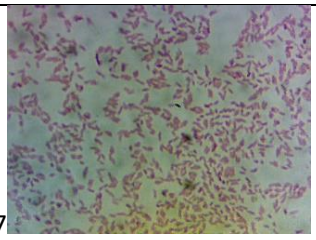
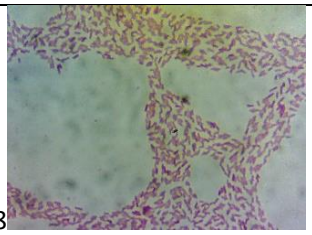
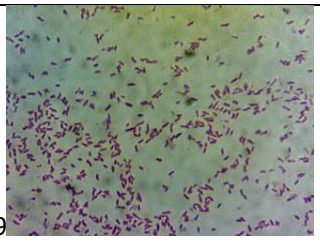
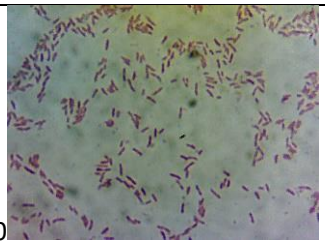
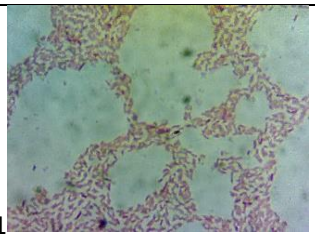
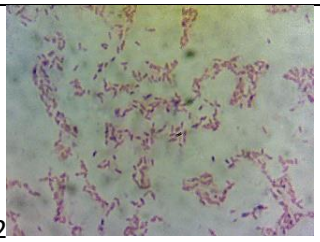
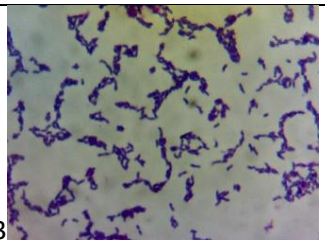
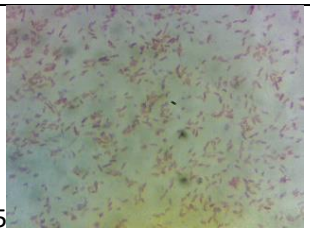
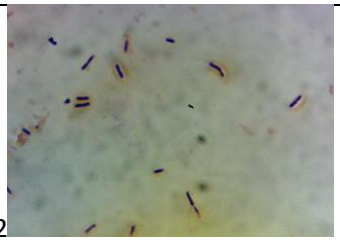
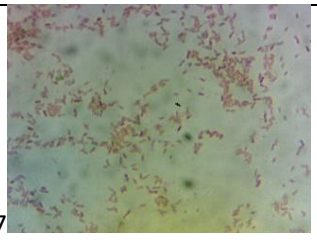
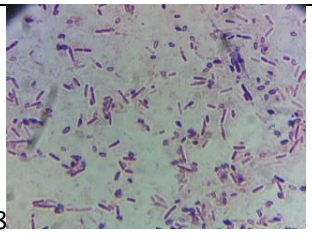
D8 	D9 	D10 
D11 	D12 	D13 
D14 	D15 	D16 
D17 	D18 	D19 
D20 	D21 	D22 
D23 	D24 	D25 
D2 	D27 	D28 

Table A11: Biochemical characteristics of the isolates of cultured rhizosphere bacteria from both media (RIM and DRSM)

SAMPLE	Catalase	Oxidase	Anti- AM	Anti-VA	Starch	MR	VP	Nitrate	Citrate	Gelatin	Urea	phosphate	Motility	Indole	H_2S	N_2-fixation	Bacteria Spp
R1	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	++	
R2	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	
R4	+	+	-	-	-	-	-	N2	+	+	-	+	-	-	++	++	*Pseudomonas sp*
R5	+	+	NA	+	+	-	-	-	-	-	-	-	+	-	-	++	*Actinobacteria sp*
R6	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	
R7	+	-	+	+	+	-	-	-	-	-	-	-	+	-	++	-	*Actinobacteria sp*
R8	+	-	-	-	-	-	-	NO2	-	-	-	-	-	-	-	+	
R11	+	+	-	-	-	-	-	N2	+	-	-	+	+	-	-	+	*Pseudomonas sp*
R12	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	
R13	+	+	-	-	-	-	-	N2	+	+	-	+	+	-	-	++	*Pseudomonas sp*
R14	+	+	-	-	-	-	-	N2	+	+	-	+	-	-	-	++	*Pseudomonas sp*
D1	-	-	+	+	-	-	-	-	-	-	+	-	+	-	-	++	
D2	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	++	
D3	+	+	-	+	+	-	-	-	-	+	-	-	+	-	-	+	
D4	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	
D5	+	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	*Pseudomonas sp*
D6	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	
D7	+	-	+	+	-	-	-	-	-	+	+	-	-	-	-	+	*Staphylococcus sp*
D8	+	-	+	+	-	-	-	NO2	+	-	+	-	-	-	-	+	
D9	+	-	+	+	-	-	-	NO2	-	-	-	-	-	-	-	-	*Staphylococcus sp(roseus)*
D10	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	
D11	+	-	+	+	+	-	-	NO2	+	-	-	-	-	-	++	+	*Actinobacteria sp*
D12	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	
D13	+	-	+	+	+	-	-	NO2	+	+	-	-	+	-	-	+	*Bacillus cereus*
D14	+	-	-	+	+	-	-	NO2	-	-	-	-	-	-	-	-	
D15	+	-	+	+	+	-	-	NO2	+	+	-	-	+	-	+	+	*Bacillus cereus*
D16	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	
D17	-	+	-	-	-	-	-	N2	+	-	-	+	-	-	+	++	*Pseudomonas sp*

D18	+	+	-	-	-	-	-	NO2	+	+	-	+	-	-	+	++	*Pseudomonas sp*
D19	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	++	*Pseudomonas sp*
D20	+	+	-	-	-	-	-	N2	+	+	-	+	-	-	+	++	*Pseudomonas sp*
D21	+	+	-	-	-	-	-	N2	+	+	-	+	-	-	++	++	*Pseudomonas sp*
D22	-	-	+	+	-	-	-	NO2	-	+	-	-	-	-	-	-	
D23	+	-	+	+	-	-	-	-	+	+	-	-	+	-	-	++	*Bacillus sp*
D24	-	-	+	+	+	-	-	NO2	+	-	-	-	-	-	-	++	*Actinobacteria sp*
D25	+	+	-	+	-	-	-	-	+	-	-	-	+	-	+	+	*Pseudomonas sp*
D26	+	-	+	+	+	-	-	NO2	+	+	-	-	+	-	-	+	
D27	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	*Pseudomonas sp*
D28	+	+	NA	+	+	+	-	NO2	-	-	-	-	++	-	-	+	*Bacillus subtilis*